CHROMATOGRAPHIC SCIENCE SERIES VOLUME 102

High Performance Liquid Chromatography in Phytochemical Analysis

Edited by
Monika Waksmundzka-Hajnos
Joseph Sherma

CRC Press
Taylor & Francis Group

High Performance Liquid Chromatography in Phytochemical Analysis

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99. Thin Layer Chromatography in Phytochemistry, *edited by Monika Waksmundzka-Hajnos, Joseph Sherma, and Teresa Kowalska*
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102. High Performance Liquid Chromatography in Phytochemical Analysis, *Monika Waksmundzka-Hajnos and Joseph Sherma*

High Performance Liquid Chromatography in Phytochemical Analysis

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CRC Press
Taylor & Francis Group
Boca Raton London New York

CRC Press is an imprint of the
Taylor & Francis Group, an **informa** business

CRC Press
Taylor & Francis Group
6000 Broken Sound Parkway NW, Suite 300
Boca Raton, FL 33487-2742

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CRC Press is an imprint of Taylor & Francis Group, an Informa business

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Printed in the United States of America on acid-free paper
10 9 8 7 6 5 4 3 2 1

International Standard Book Number: 978-1-4200-9260-8 (Hardback)

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Library of Congress Cataloging-in-Publication Data

High performance liquid chromatography in phytochemical analysis / editors, Monika Waksmundzka-Hajnos, Joseph Sherma.
p. cm. -- (Chromatographic science series ; 102)
Includes bibliographical references and index.
ISBN 978-1-4200-9260-8 (hardcover : alk. paper)
1. Phytochemicals--Analysis. 2. High performance liquid chromatography. I. Waksmundzka-Hajnos, Monika. II. Sherma, Joseph.

QK865.H578 2010
572'.2--dc22 2010034916

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In Memory of
Professor Andrzej Waksmundzki
(1910–1998)
Pioneer of chromatography in Poland

Contents

Secondary Metabolites — Isoprenoids

Secondary Metabolites — Amino Acid Derivatives

Secondary Metabolites — Compounds Derived from Acetogenine (Acetylocoenzyme A)

Preface

This is the first book to be published that gives a complete description of the techniques, materials, and instrumentation of column high performance liquid chromatography (HPLC) and its application to essentially all primary and secondary plant metabolites. Together with the companion volume, *Thin Layer Chromatography in Phytochemistry* that we coedited with Professor Teresa Kowalska in 2008 (Volume 99 in the Chromatographic Science Series edited by Dr. Jack Cazes and published by CRC Press/Taylor & Francis), the two most important analytical approaches used in phytochemical analysis have now been comprehensively covered.

The book is organized into two parts comprising a total of 34 chapters. Part I (15 chapters) begins with a chapter giving an overview of the field of phytochemistry and the organization of the book. This is followed by three chapters on the role of HPLC in the analysis of herbal drugs, quality control of plant products in dietary supplements, and chemosystematics, and then a chapter on phytochemistry, pharmacology, and the biological role of plant metabolites. Part I is completed by a series of chapters dedicated to different modes and techniques of HPLC analysis: sample preparation; stationary phases and columns; separation of nonionic compounds by normal- and reversed-phase HPLC; separation of ionic compounds by reversed-phase, ion-pair, ion-exchange, and ion-exclusion HPLC; gradient elution and computer-assisted methods; HPLC/mass spectrometry; HPLC/nuclear magnetic resonance spectrometry; HPLC with photodiode array and other detection methods; quantitative analysis and method validation; and HPLC chiral analysis.

Part II (19 chapters) contains chapters on the HPLC separation, identification, and quantification of particular classes of compounds in a great variety of sample types (plants, plant extracts, plant-derived products, etc.), starting with chapters on primary metabolites (carbohydrates, lipids, and amino acids, peptides, and proteins) and followed by those on secondary metabolites (shickimic acid derivatives, coumarins, flavonoids, lignans, isoprenoids, diterpenes, tripenes, polyterpenes, steroids, iridoids, amino acid derivatives, isoquinoline alkaloids, tropane alkaloids, other biosynthetic alkaloids, polyacetylenes, and quinoids).

Chapters were written by authors with great experience and knowledge in HPLC phytochemical analysis who are working in Austria, Bulgaria, the Czech Republic, Denmark, Finland, France, Greece, Hungary, India, Italy, New Zealand, Poland, Switzerland, Turkey, the United Kingdom, and the United States, which guarantees the book is authoritative and presents an international view of the field. The authors were given some latitude to present their material with text, tables, figures, and references in the manner they believed was best suited to produce the most practical and useful chapters for a wide range of readers with different experience levels and job descriptions.

This book will serve as a laboratory manual, reference book, or source of teaching material for undergraduate and graduate courses in chemistry, biology, and plant science, and it will be an invaluable source of information in research and analytical laboratories associated with universities, dietary supplement production, or governmental regulation. We would be pleased to hear from readers of our book about how it was helpful in their work, as well as notification of any errors and suggestions for changes, additions, and/or deletions if a second edition of the book is published.

We thank Dr. Cazes and Senior Editor Barbara Glunn and Production Coordinator Patricia Roberson of CRC Press/Taylor & Francis for their complete support of this book project from the proposal through publication.

JS would like to express appreciation to President Daniel H. Weiss and Provost Wendy L. Hill for the continuing support by Lafayette College of his research and publication activities as an emeritus professor.

Monika Waksmundzka-Hajnos
Joseph Sherma

Editors

Monika Waksmundzka-Hajnos received a PhD in analytical chemistry from the Faculty of Chemistry of Maria Curie-Skłodowska University in Lublin, Poland, in 1980. She is currently professor of pharmacy and head of the Department of Inorganic Chemistry at the Faculty of Pharmacy of the Medical University of Lublin in Lublin, Poland. Her interests involve the theory and application of liquid chromatography, taking into consideration the optimization of chromatographic systems for separation of natural mixtures and plant extracts for analytical and preparative purposes. Another scientific interest of Dr. Waksmundzka-Hajnos involves the optimization of liquid–solid processes for the extraction of biologically active secondary metabolites from plant material and the optimization of purification processes by liquid–liquid extraction (LLE) and solid-phase extraction (SPE) of crude plant extracts from ballast substances before high performance liquid chromatography (HPLC) or thin layer chromatography (TLC).

Professor Waksmundzka-Hajnos is author or coauthor of more than 120 papers and approximately 250 conference papers. She has published review articles in journals such as the *Journal of Chromatography A*, the *Journal of Chromatography B*, and the *Journal of Liquid Chromatography and Research Trends* (India). She has also authored a chapter on preparative planar chromatography of plant extracts in the textbook *Preparative Layer Chromatography*, edited by Teresa Kowalska and Joseph Sherma. Professor Waksmundzka-Hajnos coedited with Professor Kowalska and Professor Sherma the book *Thin Layer Chromatography in Phytochemistry* (published as Volume 99 in the Chromatographic Science Series by CRC Press/Taylor & Francis), and she coedited with Professor Sherma the current book, *HPLC in Phytochemical Analysis*. Dr. Waksmundzka-Hajnos has received five awards from the Ministry of Health in Poland and two awards from the Polish Pharmaceutical Society for her scientific achievements.

Dr. Waksmundzka-Hajnos has taught courses in inorganic chemistry to pharmacy and medical chemistry students for more than 30 years. She has also taught courses in instrumental analysis to students of pharmacy. Over the past 17 years, Dr. Waksmundzka-Hajnos has directed programs for over 40 MSc pharmacy students involved in the theory and practice of different liquid chromatographic techniques. She has also supervised four PhD students researching separation science.

Since 2005 Dr. Waksmundzka-Hajnos has been a member of the editorial board of *Acta Chromatographica*, the annual periodical published by the University of Silesia, Katowice, and by Akademiai Kiado, and since 2008 she has been an editor of that journal. Also since 2008, she has been a member of the editorial board of the *Journal of Planar Chromatography-Modern TLC*. She has also devoted her time to the development of many chromatographic and hyphenated techniques.

Joseph Sherma received a BS in chemistry from Upsala College in East Orange, New Jersey, in 1955 and a PhD in analytical chemistry from Rutgers, the State University, in New Brunswick, New Jersey, in 1958. He is currently the John D. and Frances H. Larkin Professor Emeritus of Chemistry at Lafayette College, Easton, Pennsylvania. Professor Sherma taught courses in analytical chemistry for more than 40 years, was head of the Chemistry Department for 12 years, and continues to supervise research students at Lafayette. He has authored, coauthored, edited, or coedited more than 720 publications, including research papers and review articles in more than 50 different analytical chemistry, chromatography, and biological journals; many invited book chapters; and more than 65 books and manuals in the areas of analytical chemistry and chromatography.

In addition to his research in the techniques and applications of thin layer chromatography (TLC), Professor Sherma has a very productive interdisciplinary research program in the use of analytical chemistry to study biological systems with Bernard Fried, Kreider Professor Emeritus of Biology at

Lafayette College, with whom he has written the book *Thin Layer Chromatography* (1st–4th editions) and edited the *Handbook of Thin Layer Chromatography* (1st–3rd editions), both published by Marcel Dekker, Inc., as well as editing *Practical Thin Layer Chromatography* for CRC Press. Professor Sherma wrote, with Dr. Gunter Zweig, a book on paper chromatography and coedited with him 24 volumes of the Handbook of Chromatography series for CRC Press and 10 volumes of the series Analytical Methods for Pesticides and Plant Growth Regulators for Academic Press. After Dr. Zweig's death, Professor Sherma edited five additional volumes of the chromatography handbook series and two volumes in the pesticide series. The pesticide series was completed under the title Modern Methods of Pesticide Analysis for CRC Press with two volumes coedited with Dr. Thomas Cairns. Three books on quantitative TLC and advances in TLC were edited jointly with the late Professor Joseph C. Touchstone for Wiley-Interscience. For CRC Press/Taylor & Francis, Professor Sherma coedited with Professor Teresa Kowalska *Preparative Layer Chromatography* and *Thin Layer Chromatography in Chiral Separations and Analysis*, coedited with Professor Kowalska and Professor Monika Waksmindzka-Hajnos *Thin Layer Chromatography in Phytochemistry*, and coedited with Professor Waksmundska-Hajnos the current book, *HPLC in Phytochemistry.*

Professor Sherma served for 23 years as the editor for residues and trace elements of the *Journal of AOAC International* and is currently acquisitions editor of that journal, and he is now on the editorial advisory boards of the *Journal of Liquid Chromatography and Related Technologies*; the *Journal of Environmental Science and Health (Part B)*; the *Journal of Planar Chromatography-Modern TLC*; *Acta Chromatographica*; *Acta Universitatis Cibiniensis, Seria F. Chemia*; and *Current Pharmaceutical Analysis.* Professor Sherma has for 12 years guest-edited with Professor Fried, annual special issues on TLC for the *Journal of Liquid Chromatography and Related Technologies*, and he regularly guest-edits special sections in the *Journal of AOAC International* on specific subjects in all areas of analytical chemistry. He has also written for 11 years an article on different aspects of modern analytical instrumentation for each issue of the *Journal of AOAC International*. Professor Sherma has written the biennial review of planar chromatography for the American Chemical Society (ACS) journal *Analytical Chemistry* continually since 1970. He was the recipient of the 1995 ACS Award for Research at an Undergraduate Institution sponsored by Research Corporation.

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Part I

1 Overview of the Field of High Performance Liquid Chromatography in Phytochemical Analysis and the Structure of the Book

Monika Waksmundzka-Hajnos and Joseph Sherma

CONTENTS

1.1 SURVEY OF PHYTOCHEMISTRY

Phytochemistry, or the chemistry of plants, one of the early subdivisions of organic chemistry, has been of great importance in the identification of plant substances. In a strict sense, phytochemistry is the study of phytochemicals, which are chemicals derived from plants. In a narrower sense, the term is often used to describe the large number of primary and secondary metabolic compounds found in plants. Many of these are synthesized in plant tissues to provide protection against insect attacks, plant diseases, ultraviolet (UV) radiation, environmental contaminants, and so on. Plant metabolites also exhibit a number of protective functions for human consumers. With the development of new phytochemical methods, additional information has become available for use in conjunction with such research disciplines as plant physiology, plant biochemistry, plant taxonomy, plant biotechnology, and pharmacognosy.

Plant physiology is a discipline concerned with the function, or physiology, of plants. Fundamental processes such as photosynthesis, respiration, plant nutrition, plant hormone functions, tropisms, nastic movements, photoperiodism, photomorphogenesis, circadian rhythms, environmental stress physiology, seed germination, dormancy and stomata function and transpiration, and plant water relationships are studied by plant physiologists. For phytochemical investigations connected with plant physiology, the response of plants to various external factors or environmental conditions is very important and is revealed in the synthesis of secondary metabolites in plant tissues.

Knowledge of the entire plant biochemistry field ranges from photosynthesis (the synthesis of natural plant products) to all kinds of genetic engineering with its many commercial applications. Topics include cell structure and function; primary lipid and polysaccharide metabolism; nitrogen fixation; phloem transport; synthesis and function of isoprenoids, phenylpropanoids, and other secondary metabolites; and plant growth regulation and development. Plant biochemistry field provides a description of photosynthesis, primary and secondary metabolism, the function of phytohormones, and molecular engineering.

Chemosystematics, also called chemotaxonomy, can be viewed as a hybrid science that complements available morphological data to improve plant systematics. Phytochemical constituents (especially the amino acid sequences of common plant proteins) can be used to characterize, describe, and classify species into taxa. Interest in this aspect of systematics has increased with development of rapid, accurate, and precise analytical techniques, and data from as many sources as possible can be employed in plant classification. Evidence from chemical constituents has already led to the reconsideration of many plant taxa. For example, a number of taxonomically difficult families have been successfully grouped on the basis of their secondary metabolite profiles.

The most important application of phytochemical investigation methods is to the field of pharmacognosy. Pharmacognosy is the study of medicines derived from natural sources (mainly from plant materials). The history of phytotherapy is almost as long as the history of civilization. The term *pharmacognosy* was used for the first time by the Austrian physician Schmidt in 1811; it is derived from the Greek words *pharmakon* ("drug") and *gnosis* ("knowledge"). Originally—during the nineteenth century and the beginning of the twentieth century—"pharmacognosy" was used to define the branch of medicine or *commodity sciences* that dealt with drugs in their crude, or unprepared, form. Crude drugs are the dried, unprepared material of plant, animal, or mineral origin used for medicine. Although most pharmacognostic studies focus on plants and medicines derived from plants, other types of organisms are also regarded as pharmacognostically interesting, in particular, various types of microbes (bacteria, fungi, etc.) and, recently, various marine organisms. The contemporary study of pharmacognosy can be divided into the fields of medical ethnobotany, the study of the traditional use of plants for medicinal purposes; ethnopharmacology, the study of the pharmacological qualities of traditional medicinal substances; phytotherapy, the study of the medicinal use of plant extracts; and phytochemistry, the study of chemicals derived from plants (including the identification of new drug candidates derived from plant sources).

Phytotherapy is the study of the use of extracts from natural origin as medicines containing health-promoting agents. In modern science it appeared in the nineteenth century when the first biologically active alkaloids (morphine, strychnine, narcotine, caffeine, etc.) were isolated from plants. In the 1930s, chemotherapy began with the appearance and therapeutic usage of synthetic sulfonamides and antibiotics. The lack of studies proving the effects of herbs used in traditional care is especially an issue in the United States, where herbal medicines have fallen out of use since World War II and have been considered suspect since the Flexner Report of 1910 led to the closing of the eclectic medical schools where botanical medicine was exclusively practiced. Furthermore, most herbal studies in the latter part of the twentieth century were published in languages other than English. As it may be more difficult to review foreign-language publications, many of these publications have been incorporated into the U.S. Food and Drug Administration (FDA) determinations of drug safety. In 1994, the U.S. Congress passed the Dietary Supplement Health and Education Act (DSHEA) regulating labeling and sales of herbs and other supplements. Most of the 2000 U.S. companies making herbal or natural products choose to market their products as food supplements, which do not require substantial testing. With the development of chemotherapy, it turned out that synthetic drugs exert—along with the positive therapeutic effect—also harmful and often irreversible side effects. Because of this, in recent years a return to phytotherapy has been observed. This return has been further spurred by an appeal of the World Health Organization to screen plant material for biologically active compounds contained therein and their effects, such as pronounced anticancer activity. It is firmly believed that a great, yet still not fully revealed, therapeutic potential exists in plants, because so far only a few percent out of the estimated 250,000 plant species have been investigated with regard to their usefulness in medicine.

Nowadays, many medicines of natural origin are appreciated for their high effectiveness and low toxicity, and they are widely used commercial products. Plant materials are often obtained from natural sources, although many of the medicinal plants are also cultivated. In view of these facts, there is a high and increasing need for efficient purity control of plant material, and further for assessment of their identity and chemical composition, in order to obtain the expected therapeutic effect.

In herbal medicine, standardization refers to providing processed plant material that meets a specified concentration of a specific marker constituent. Active constituent concentrations may be misleading measures of potency if cofactors are not present. A further problem is that the important constituent is often unknown. For instance, Saint John's wort is often standardized with respect to the antiviral constituents hypericin or hyperforin or both, although there may be some 24 known possible constituents. Only a minority of chemicals used as standardization markers are known to be active constituents. The process of standardization is not yet carried out consistently: Different companies use different markers, different levels of the same markers, or different methods of testing for marker compounds.

Quality in the use of crude drugs or plant medicines depends on a variety of factors: genetically strong seed; correct species; maturity of the plant at harvest; good soils; air quality; climate; organoleptic factors such as intensity of color, flavor, and odor; processing after harvest; and a variety of others. These conditions have been noted in historical herbals, and this was standard pharmacognosy curriculum for many years. Storage after collection is also a factor worthy of study. In modern times the foregoing aspects are no less important, but they have been neglected with the advent of laboratory testing, although it generally is true that only certain constituents are identified and measured. Processes like column high performance liquid chromatography (HPLC), gas chromatography (GC), ultraviolet/visible (UV/VIS) spectrometry, or atomic absorbance spectrometry (AAS) are used to identify species, measure bacteriological contamination, assess potency, and eventually create certificates of analysis for the material. Quality should be overseen by either authorities ensuring good manufacturing practices (GMPs) or regulatory agencies such as the FDA. In the United States, one frequently sees comments that herbal medicines are unregulated, but this is not correct, since the FDA and GMP regulations are in place. In Germany, the Commission E has produced a book of German legal-medical regulations that include quality standards.

The political issues surrounding the safety of crude drugs vary from considering natural remedies "safe" regardless of potential dangers to considering them a dangerous unknown. Ephedra has been known to have numerous side effects, including severe skin reactions, irritability, nervousness, dizziness, trembling, headache, insomnia, profuse perspiration, dehydration, itchy scalp and skin, vomiting, hyperthermia, irregular heartbeat, seizures, heart attack, stroke, or death. Poisonous plants that have limited medicinal effects are often not sold in material doses in the United States or are available only to trained practitioners. These include *Aconite, Arnica, Belladonna, Bryonia, Datura, Gelsemium, Henbane, Male Fern, Phytolacca, Podophyllum,* and *Veratrum.* Secondly are herbs like *Lobelia, Ephedra,* and *Euonymus* that cause nausea, sweating, and vomiting, which were traditionally prized for this action. Third are plants such as comfrey and *Petasites* with specific toxicity due to hepatotoxic pyrrolizidine alkaloids. There are other plant medicines that require caution or can interact with medications, including Saint John's wort or grapefruit.

Most bioactive compounds of natural origin are secondary metabolites, that is, species-specific chemical agents that can be grouped into various categories. A typical protocol to isolate a pure chemical agent of natural origin is bioassay-guided fractionation (BAGF), meaning step-by-step separation of extracted components based on differences in their physicochemical properties, and assessment of the biological activity, followed by another round of separation and assay. Typically, such work is initiated after a given crude drug formulation (normally prepared by solvent extraction of the natural material) is deemed "active" in a particular in vitro assay. If the end goal of the work at hand is to identify which of the scores or hundreds of compounds are responsible for the observed in vitro activity, the path to that end is fairly straightforward: (1) fractionate the crude extract, for example, by solvent partitioning or chromatography; (2) test the fractions thereby generated with in vitro assays; (3) repeat steps 1 and 2 until pure, active compounds are obtained; and (4) determine structure(s) of active compound(s), typically by using spectrometric methods. The most common means for fractionation are solvent–solvent partitioning and chromatographic techniques such as HPLC, medium-pressure liquid chromatography, flash chromatography, open-column chromatography, vacuum liquid chromatography (VLC), and thin-layer chromatography (TLC), with each technique being most appropriate for a given amount of starting material. Countercurrent

chromatography (CCC) is particularly well suited for BAGF. After isolation of a pure substance, the task of elucidating its chemical structure can be addressed. For this purpose, the most powerful methodologies available are nuclear magnetic resonance (NMR) spectrometry and mass spectrometry (MS). In the case of drug-discovery efforts, structure elucidation of all components that are active in vitro is typically the end goal. In the case of phytotherapy research, the investigator may use in vitro BAGF as a tool to identify pharmacologically interesting or important components of the crude drug. The work does not stop after structural identification of in vitro active substances, however. The task of "dissecting and reassembling" the crude drug one active component at a time, in order to achieve a mechanistic understanding of how it works in phytotherapy, is quite daunting. This is because it is simply too difficult, from cost, time, regulatory, and even scientific perspectives, to study experimental fractions of the crude drug in humans. In vitro assays are, therefore, used to identify chemical components of the crude drug that may reasonably be expected to have a given pharmacological effect in humans and to provide a rational basis for standardization of a crude drug formulation to be tested in humans.

Plant materials, galenic preparations, and isolated compounds proposed for therapy have to meet certain strictly determined standards. With the most important materials, these standards are simply the pharmacopoeia requirements, although a vast number of herbs used in formal and popular medicines are not included in any pharmacopoeia. Standardization of the plant material and of herbal preparations is meant to guarantee their therapeutic value, and it is a result of the investigations on biologically active components.

1.2 PROCEDURES OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Among the chemical methods used for plant examination, chromatographic analysis plays a very important role, and it has been introduced to all modern pharmacopoeias. Due to numerous advantages of the chromatographic methods (such as their specificity and the possibility to use them for sensitive qualitative and quantitative analysis), they comprise an integral part of medicinal plant analysis.

The following chromatographic methods are most frequently applied in phytochemical analysis: one- and two-dimensional paper chromatography, one- and two-dimensional TLC, HPLC, GC, and CCC. These methods can also be used for isolation of the individual components from the component mixtures on a preparative and micropreparative scale.

HPLC is a chromatographic technique widely used for qualitative and quantitative analysis of organic compounds present in multicomponent mixtures, such as natural plant extracts. It utilizes a fully automated instrumental system including a column, mobile-phase (eluent) container, mobile-phase pump, injector, and detector. The HPLC system is controlled by a computer program that registers chromatographic profiles and all data of the individual peaks: retention time, peak height, peak width, surface area of a peak, system efficiency, peak symmetry factor, and so on. Because the column providing the separation is connected to the detector, HPLC allows detection and on-line identification of a wide range of organic and inorganic compounds.

The refractive index (RI) detector is an example of a universal detector used in HPLC. However, it is rarely used because all mobile-phase solvents and additives show a significant refractive index response, which makes the use of gradient elution impossible. Other factors, such as the need for temperature control, the effect of dissolved gases in the mobile phase, and low sensitivity, also limit the use of the RI detector for many routine applications. The second type of universal detector is based on evaporative light scattering (ELS). Use of the ELS detector is restricted to nonvolatile analytes and volatile mobile phases.

The detector type most often applied in HPLC is the UV/VIS photodiode array detector (DAD). DAD UV detectors allow simultaneous collection of chromatograms over a range of wavelengths during a single run. Therefore, the DAD provides more information on sample composition than is

provided by use of a single wavelength detector. The UV spectrum of each separated peak is also an important tool for selecting an optimum wavelength to verify peak purity and peak identity. The latter possibility allows comparison of the UV spectra for a standard compound with a sample peak having the same retention time as the standard. The DAD can also be used to examine the chromatograms at different wavelengths, which enables group classification (e.g., taxoids have an absorption maximum at 230 nm, and at 245 nm they absorb poorly). However, UV detection is limited to compounds having chromophore groups (e.g., aromatic rings), and it is not suitable for the compounds that do not absorb in the UV range.

The more selective and sensitive fluorescence (FL) detector is also used in HPLC. Sensitivity of FL detectors is typically three orders of magnitude higher compared to UV detectors, and the detector response is linear over about two or three orders of magnitude for typical conditions. Selectivity is higher because either or both of the excitation and emission wavelengths can be changed. However, only a few analytes possess natural fluorophores. Because of that, derivatization is often necessary prior to use of this detector. Another selective detector used in HPLC is the electrochemical (EC) detector, which operates on the principle of the direct-current amperometry (DCA) or conductivity. These detection methods are applied to compounds that exhibit electrochemical activity. In this case, however, derivatization is also needed to convert the nonelectrochemically active analytes into active ones. Apart from the fact that the analyte must be electrochemically active, the EC detector requires temperature control. This fact and the need for solvents with very high purity, stabilized electrical signals, and so on hampers the use of this detector.

The use of a mass spectrometer as an HPLC detector is becoming commonplace for the qualitative and quantitative analysis of mixture components. MS fragmentation patterns can be used to identify each peak. For all MS techniques, the analyte is first ionized in the source, since MS can detect only charged species. Ions having discrete mass/charge ratios (m/z) are then separated and focused in the mass analyzer and detected by the detector.

Combination of chromatographic separation techniques with NMR spectrometry is one of the most powerful and time-saving methods for the separation and structural elucidation of unknown compounds and mixtures. An on-line HPLC-NMR tandem system has important advantages, especially for structure elucidation of light- and oxygen-sensitive substances, such as hop bitter acids and carotenoid stereoisomers. In such cases, structure elucidation with HPLC-MS is not possible because two isomers often exhibit the same fragmentation pattern. Using the classical method consisting of off-line separation followed by enrichment and transfer to an NMR spectrometry sample tube would result in isomerization of the isolated substances. A closed-loop HPLC-NMR flowthrough system solves this problem. The on-line HPLC-NMR technique also allows continuous registration of the time changes as they appear in the chromatographic run. Unequivocal structural assignment of unknown chromatographic peaks is possible in two-dimensional stopped-flow HPLC-NMR experiments.

Less commonly used in HPLC are polarimetric detectors, used to detect enantiomers, and infrared and radioactivity detectors.

A main element of HPLC is the column used for the separation processes. Because columns can be used for long periods of time, durable, and well-performing columns are required. For this reason, sorbents used as HPLC packings should reversibly interact with analytes and the mobile-phase components. This means that specific interactions (ion–ion, dipole–ion, etc.) should not occur because they cause deactivation of the column packing and a resulting decrease in efficiency and separability in the course of usage. Chemically bonded stationary phases with nonpolar alkyl or phenyl ligands or with medium-polarity ligands (containing the CN, NH_2, and diol functionalities immobilized on the silica matrix) are mainly used today in the column separations. Application of porous polymers, graphitized carbon, silica, alumina, and the zirconia-based HPLC column packings is also noteworthy.

Reversed-phase (RP) HPLC is one of the most frequently used chromatographic procedures. Today, approximately 80% of all chromatographic separations are performed using nonpolar

chemically bonded phases, containing mainly octadecylsilyl chains (C_{18}). This results from the universality of these phases, relatively low analysis costs, and the ease of the procedure. In such systems, the mobile phase consists of water and organic modifiers (such as methanol, acetonitrile, tetrahydrofuran, dioxane, etc.), and sometimes extra additives (e.g., buffers of different pH values, ion-pair reagents, ion suppressants, or silanol blockers). These mobile-phase additives are applied for separations of organic electrolytes. RP-HPLC can be performed either by isocratic elution (with a mobile phase having constant composition) or by gradient elution (with changes of the mobile-phase strength resulting from variation in composition in the course of a chromatographic run). Gradient elution is usually obtained by gradual addition of a high-elution-strength solvent to a low-elution-strength solvent (usually water in RP-HPLC). Gradient elution lowers the analysis time and improves separation efficiency. The gradient elution mode is applied to mixtures of the components differing considerably in polarity, when the general elution problem occurs. A linear gradient is usually applied, but the gradient profile can be programmed to various shapes. Gradient elution suitable for a given separation can be optimized with the aid of special computer programs simulating separation of a particular mixture (which is characterized with known retention parameters). For separation of organic electrolytes, pH gradients can be applied, or a binary gradient of a mobile-phase modifier and pH. However, gradient elution limits the use of certain detectors and increases the time needed for equilibration of the column.

The physical properties of the packings and the size of the columns are important parameters in HPLC. Minimization of sorbent particles, increases in the specific surface area of the sorbents, and controlled porosity generally cause an increase in column efficiency and make separation even of fairly complex mixtures possible. For example, columns with a low particle diameter of 3.5 μm perform better than those with 5 μm particles. If we compare two columns of the same length, similar resolution on the column with the 3.5 μm particles is available in half the separation time, and the use of a higher mobile-phase flow rate is also possible without a loss of column efficiency. Microbore columns reduce solvent consumption. These columns are especially useful when interfacing an HPLC instrument with mass spectrometers and other instruments requiring small solvent-input volumes. This results in higher mass-detection sensitivity of analytes. In the last decade, monolithic columns have been applied to difficult separations. Silica-based monolithic columns contain a novel chromatographic support in which traditional particulate packing is replaced by a single and continuous network of porous silica (known as a monolith). The main advantage of this continuous network is decreased backpressure, due to the presence of macropores (2 μm) throughout the network. This allows high flow rates and, hence, fast analyses that cannot be obtained with the traditional particulate columns.

Sample preparation is an essential part of HPLC analysis, intended to provide a reproducible and homogeneous solution that is suitable for injection onto the column. The goal of sample preparation is a sample solution that (i) is free of interferences, (ii) will not damage the column, and (iii) is compatible with an applied HPLC method (i.e., sample solvent is soluble in the mobile phase and does not affect sample retention). Sample pretreatment is usually carried out in a manual off-line mode. However, many sample-preparation techniques have been automated with use of appropriate instrumentation. Although automation can be expensive and complicated, it is indispensable when large numbers of samples have to be analyzed and the time or labor per sample is excessive. For example, solid-phase extraction (SPE) can be interfaced with a robot to move sample containers and/or the other sample-preparation devices (balances, mixers, dilutors, autosamplers, etc.). Column switching (coupled column chromatography) can also be used as a kind of sample-preparation method. It is used not only for the complete resolution of partly separated fractions from the first system but also for the removal of the contaminants ("column killers") or the late eluters, thus extending the column life and improving resolution of a mixture of components. Column switching can be used as an on-line method of sample pretreatment in the chromatographic run.

Summing up, HPLC is the principal separation and analysis technique in plant chemistry research. It can be used in a search for standardization of plant drugs and for quantitative analysis of pharmacologically active compounds. HPLC-DAD enables peak purity control and group

identification (or preliminary identification) of the known extract components. LC-MS or LC-NMR enables identification of the known and unknown plant constituents. These are the benefits that make the chromatographic methods widely used in the search for new drugs from the plants that have been used in various ethnomedicines literally for ages. Moreover, such hyphenated techniques enable data collection from numerous plant samples, which proves very useful in chemosystematics. Similar investigations are very helpful for determination of plant drugs or dietary supplements, and of plant food origin and quality.

1.3 ORGANIZATION OF THE BOOK

This book comprises 34 chapters, divided into two parts. Part I consists of 15 chapters and provides general information on those areas of science that are related to phytochemistry and can benefit from the use of HPLC, such as the instrumentation and chromatographic systems involved. Chapter 2 focuses on herbal drugs and the role of chromatographic methods in the examination of herbs and herbal-product quality by use of fingerprinting and marker compounds for identification and standardization of botanical drugs by chromatographic methods, especially HPLC.

Chapter 3 is devoted to plant products in nutrition and dietary supplements. It provides quality aspects of botanical products applied and includes a short overview of HPLC analysis of phytochemicals (phenolics, carotenoids, phytoestrogens, terpenes, lignans, etc.) in foods (fruit, fruit juices, beverages, wines, tea cultivars, hop resins, beer, etc.) and in dietary supplements (dietary bee products; dietary supplements containing soy, *Ginkgo biloba*; etc.).

Chapter 4 focuses on the role of HPLC in chemosystematics, also called chemotaxonomy. This chapter starts with a definition of this particular branch of phytochemical science, which involves classification of plant organisms based on the qualitative and quantitative differences in their composition of primary and secondary metabolites. Then the authors highlight the areas of main interest for chemosystematic studies in various branches of science, such as botany, chemical ecology, and food sciences, and the use of HPLC in them. The advantages and limitations of such investigations are also emphasized.

Chapter 5 covers the classification, biosynthesis, and biological importance of plant metabolites, including primary metabolites (carbohydrates, lipids, amino acids, peptides, and proteins) and secondary metabolites from various biochemical pathways. The authors also take into consideration other metabolites with a complicated or mixed biosynthetic origin. The chapter discusses as well the pharmacological activities of primary and secondary metabolites and the therapeutic application of plant materials (natural drugs) in the following cases: nervous system diseases, cardiovascular system disorders, respiratory system diseases, gastrointestinal system disorders, urinary system diseases, gynecological and andrological diseases, rheumatic diseases, and dermatology. Natural compounds having antimitotic, anticancer, and immunomodulatory activities are also presented.

Chapter 6 deals with sample preparation of plant material, taking into account the preliminary processing (collecting, drying, storage, and homogenization of samples) as well as methods of extraction from plant material (traditional methods, Soxhlet extraction, and modern extraction methods such as microwave- or ultrasound-assisted methods or pressurized extraction). The authors also describe procedures applied for sample purification and concentration such as liquid–liquid extraction (LLE), crystallization, SPE, column chromatography, TLC, and membrane separations. Information is provided on methods of hydrolysis of esters, glycosides, and natural polymers and on prechromatographic derivatization.

In Chapter 7, stationary phases and columns that are particularly useful in the analysis of primary and secondary metabolites from plant extracts are described. This chapter covers virtually all columns used for this purpose, including different kinds of sorbents (silica, alumina, silica bonded alkyl phases, polymeric supports, polar bonded stationary phases, cellulose, chiral bonded phases, and ion exchange phases), particle size variations, column hardware (stainless steel and polymer), and column sizes. The chapter also presents some methodological problems connected with technical

details of chromatographic columns, such as the effect of column size on separation and efficiency, the column life of different chromatographic systems, conditions, kinds of samples, and so on.

The two following chapters deal with the development of methods for separation of plant metabolites. Chapter 8 is devoted to optimization of the separation of nonionic analytes by RP and normal-phase (NP) HPLC in isocratic and gradient elution modes. The authors also present applications of particular systems in phytochemical analysis. Chapter 9 deals with the methods of separation of ionic analytes involving RP systems with aqueous buffered mobile phases at various pHs containing ion suppressants or silanol blockers. Ion-pair systems are described, including the effect of mobile-phase pH and buffer type as well as ion-pair type and concentration on the separation of organic electrolytes. The development of methods of ion exchange and ion exclusion chromatography for the separation of ionic analytes is also presented.

Chapter 10 focuses on gradient elution methods and reasons for their application. Multidimensional systems and the use of mixed stationary phases are presented. In this chapter, computer-assisted method development is emphasized.

The next two chapters present methods of identification and/or quantification of plant metabolites by hyphenated techniques such as LC-MS and LC-NMR. Chapter 11 discusses principles of on-line hyphenation of HPLC to MS, including analytical-scale chromatography and miniaturized chromatographic systems, as well as off-line LC-MS. Applications for structural elucidation of analytes with examples for quantification of selected analytes and in metabolomics are included. Chapter 12 characterizes the principles of LC-NMR by direct and indirect hyphenation as well as problems connected with the compatibility of both techniques. Applications of LC-NMR in plant analysis are presented, as well as limitations of on-line methods.

Chapter 13 deals with the detection methods of analytes applied on-line in the liquid chromatograph. Detection methods other than MS and NMR are presented for use in quantitative and quantitative analysis. Detector requirements, criteria, and properties of detectors are described, along with selected applications of the UV/VIS DAD, RI, FL, Fourier transform infrared (FT-IR), Ramon Spectroscopy (RS), photothermal, conductivity, ELS, radioactivity, and polarimetry detectors.

Chapter 14 describes quantitative analysis by external standardization, internal standardization, and other methods as well as method validation in HPLC, which includes detection and quantification limits, accuracy, precision, ruggedness/robustness, linearity, specificity, and so on.

Requirements for chirality confirmation of some natural plant products by HPLC are the subject of Chapter 15. It describes mechanisms of chiral separations in NP and RP systems and presents applications of these systems in plant analysis.

Part II of the book is divided into subsections that reflect the types of metabolites that occur in plants. Chapters 16–18 refer to primary metabolites.

Chapter 16 deals with the chemistry of carbohydrates, and their classification and occurrence in the plants as mono-, oligo-, and polysaccharides; glycoconjugates; and aminoglycosides. It provides a historical overview of carbohydrate HPLC and the recommended analytical methods, including sample preparation and the most suitable HPLC systems for the analysis of particular carbohydrate groups.

In Chapter 17, analyses of different classes of plant lipids are presented. The chapter focuses on problems associated with the detection of lipids by HPLC and isolation of lipids from natural sources. Then the authors present chromatographic methods and systems used for separation of nonpolar lipids, polar lipids including phospholipids, glycolipids, and proteolipids.

Chapter 18 focuses on free amino acids, peptides, and proteins, including their occurrence in plants and the use of HPLC to separate the individual groups of these compounds. The authors also present methods for protein HPLC analysis and their importance in taxonomic studies.

The next part of the book deals with the secondary metabolites occurring in plant tissues. It is divided into sections according to the metabolic pathways in which individual substances are synthesized.

Chapter 19 starts with the phenolic compounds that belong to the metabolic pathway of shickimic acid, that is, with phenols, phenolic acids, and tannins. It describes the structure, physicochemical properties, and classification of these compounds; their biological importance; sample-preparation methods; and the various HPLC systems and coupled techniques that are used for their separation and qualitative and quantitative analysis.

Chapter 20 deals with coumarins that belong to the phenol class and are also derived from shickimic acid. Details are provided on a general overview, biological activity of coumarins, and application of HPLC to coumarin analysis by NP, RP, and hyphenated techniques.

Chapter 21 is dedicated to the phenolic compounds originating from a similar pathway as coumarins, that is, to flavonoids. After a short introduction on the chemistry, biochemistry, and medical significance of flavonoids, the methods for their HPLC analysis are described. The applications of stationary phases and columns, mobile-phase systems and modes, and detectors are discussed. Multidimensional systems and hyphenated techniques for flavonoid analysis are also presented.

The section of the book on secondary metabolites ends with lignans, also originating from shickimic acid. Chapter 22 is focused on the chemistry, occurrence in plant material, and pharmacological activity of representatives of this group, followed by procedures for sample preparation and the HPLC analysis. Details about chromatographic systems for NP, RP, and chiral separations of lignans in herbal extracts, preparations, and biological fluids are also reported.

The next section of the book is focused on isoprenoid derivatives, which include several groups of compounds. It starts with Chapter 23 on the volatile compounds (mono- and sesquiterpenes), including their definition, classification, occurrence, and importance. Then the following applications of HPLC are discussed: identification and quality control of the volatile fractions in pharmacopoeias and monographs; quality control of traditional Chinese medicines (TCMs); determination of pharmacologic and toxic plant ingredients; improvement of the cultivation of plants; quality control of food, food supplements, and cosmetics; and analysis of active compounds in mosses and fungi.

Chapter 24 covers diterpenoids and presents their structure, physicochemical properties, natural occurrence, pharmacological activity, and chemotaxonomic significance. The details of sample preparation and the analytical HPLC separations of this group of compounds with the aid of different chromatographic systems are described, including multidimensional and coupled techniques. The chapter ends with a comparison of the performance of HPLC with that of the other chromatographic and related techniques used in diterpenoid analysis.

The next group of compounds that belong to the isoprenoid metabolic pathway are triterpenes, which are described in Chapter 25. After a short introduction on the structure and properties of this group, chromatographic systems and detection methods applied in the analysis of triterpenes (including saponins) are presented.

Chapter 26 is focused on tetra- and polyterpenes; among them, carotenoids represent the most important group of compounds. First, their structure, occurrence, and properties are presented. Then the aspects of the HPLC analysis (such as detection and instability of carotenoids) are emphasized. The use of polar and nonpolar sorbents and recommended mobile phases are discussed. Practical applications and examples end the chapter.

The next large group of compounds that belong to the isoprenoid pathway are steroids, and they are presented in Chapter 27. In the introductory part of this chapter, the usefulness and validity of HPLC for the separation of steroids are described. Then an overview of the literature is provided, taking into account the classes of phytosterols, steroids (brassinosteroids, bufadienolides, cardenolides, ecdysteroids, steroidal saponins, steroidal alkaloids, vertebrate-type steroids, and withanolides), and the related triterpenoids (cucurbitacins). Structural diversity, separation systems, and the detection and quantification for each class of compounds are presented.

Iridoids are the last group of compounds that belong to the isoprenoid pathway, and they are described in Chapter 28. After an introduction to the structure and physicochemical properties of iridoids, the isolation of this group of compounds from plant material and sample preparation are

emphasized. Finally, HPLC systems and techniques applied to the analysis of iridoids are described, including hyphenated techniques.

The next four consecutive chapters deal with alkaloids synthesized in the plant organisms from amino acids. There are several groups of alkaloids differing in their structure, properties, and biological activity.

Chapter 29 focuses on indole alkaloids. First, the chemical structure, occurrence, and pharmacological and toxicological importance of this group are discussed. This preliminary information is followed by a detailed description of sample preparation and HPLC separations of indole alkaloids, including chromatographic systems, techniques, and detection methods. Details on the separations of the particular types of indole alkaloids are also presented.

Chapter 30 is devoted to the structure, properties, and biological activity of isoquinoline alkaloids. Details on separation in RP and NP systems, including detection methods for such groups as opium alkaloids and protoberberine alkaloids and derivatives, are presented. The application of LC-MS for these purposes is also described.

Tropane alkaloids are handled in Chapter 31. The chemistry and stereochemistry of tropane and related alkaloids and their biosynthesis and natural occurrence are presented first. Various methods of extraction of this group of compounds from plant material are described, followed by the pretreatment of the extracts by liquid–liquid partitioning (LLP), solid-assisted LLP (Extrelut), and SPE. Then information on HPLC of tropane alkaloids including their quantification is provided. The chapter gives detailed information on hyphenated techniques used in tropane alkaloids analysis, and a comparison is made with the results originating from the other separation techniques in use.

Chapter 32 is focused on the remaining groups of alkaloids, including phenylethylamine derivatives; quinoline derivatives (*Cinchona* alkaloids); pyrrolidine, pyrrolizidine, piridine, and piperidine derivatives (*Tobacco*, *Lobelia*, *Pepper*, *Pelletierine*, *Sedum*, *Senecio* alkaloids); quinolizidine alkaloids (*Lupine* alkaloids); xanthine; imidazole derivatives; and diterpene alkaloids. Preparation of extracts, the most frequently employed HPLC systems, and the detection methods applicable to each individual group are presented.

The last two chapters are devoted to the secondary metabolites derived from acetogenine (acetylocoenzyme A). Chapter 33 deals with polyacetylenes' distribution in plants and pharmacological activity. Sample-preparation techniques as well HPLC separation, detection, and isolation methods in various systems are described.

Chapter 34 is focused on quinones (antraquinones and naphthoquinones). Their occurrence in plants, pharmacological activity, and HPLC techniques applied to their separation and isolation are discussed.

The authors who agreed to contribute chapters to the book are all recognized international experts in their respective fields. The book will serve as a comprehensive source of information and training on the state-of-the-art phytochemistry methods performed with the aid of HPLC. It will help in analysts with method development to solve problems connected with practical separations and analyses of plant extract fractions of active metabolites.

A computer-assisted search has found no previous book on HPLC in phytochemical analysis. Three editions of the book *Phytochemical Methods* (1973, 1984, and 1998) by J.B. Harborne (Chapman and Hall, London) had chapters organized by compound type, most of which contained some information on HPLC analysis. However, these information sources are not comprehensive, and the first two are now out of date. This book will fill the void in information in the critical field of phytochemical analysis.

This new book is a companion to *Thin Layer Chromatography in Phytochemistry*, edited by Monika Waksmundzka-Hajnos, Joseph Sherma, and Teresa Kowalska, which was published by CRC Press/Taylor & Francis in their Chromatographic Science Series, edited by the late Jack Cazes. TLC and HPLC have complementary advantages that are of great value in phytochemical analysis, and it is critical to have available the complete information on techniques and applications for both methods provided by these two books.

2 Herbal Drugs and the Role of Chromatographic Methods in Their Analysis

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CONTENTS

2.1 INTRODUCTION: WHAT IS CHROMATOGRAPHY?

Chromatography is the science that studies the separation of molecules based on differences in their structures. In chromatography, a complex of compounds are separated over a stationary support, through different interactions with that support. So, according to these different (stronger or weaker) interactions with the support, this complex will move quickly or less quickly. In this way, even chemically similar molecules can be separated from each other. Chromatographic separations can be carried out using a variety of supports, including paper (paper chromatography, PC), silica on glass plates (thin-layer chromatography, TLC), volatile gases (gas chromatography, GC), and liquids, which may incorporate hydrophilic, insoluble molecules (liquid chromatography, LC) [1,2]. In all chromatographies, there is a mobile phase and a stationary one. In PC and TLC, the mobile phase is

the solvent, while the stationary phase is either a strip or piece of paper (for PC) or a thin-layer cell (for TLC). Both these kinds of chromatography use capillary action to move the solvent through the stationary phase [3,4].

2.2 CHROMATOGRAPHY: THEN AND NOW

Chromatography is a physical method that scientists use to separate organic and inorganic compounds so that they can be further analyzed and studied. The word *chromatography* derives from *chroma* (in Greek, χρώμα) and *graphy* (in Greek, γραφή) and means "color writing." The Russian botanist Mikhail Semenovich Tswett invented chromatography in 1903 and described the "chromatogram" and its development by using different eluents. Tswett's technique is now described as normal-phase chromatography [1,2,3]. In 1941, Martin and Synge invented and worked on partition chromatography (liquid–liquid chromatography [LLC]), while the planar form on sheets of filter paper impregnated with water or another liquid was also introduced by Martin and his scientific team, as well as the retardation factor and the proposal that the mobile phase could also be a gas, which later led to GC [5,6].

2.3 OVERVIEW OF CHROMATOGRAPHY TECHNIQUES

2.3.1 The Different Types of Chromatography

Four types of chromatographic techniques are routinely used:

- Paper chromatography
- Thin-layer chromatography
- Column chromatography
- Gas chromatography

Paper chromatography (PC) is one of the most common types of chromatography. Here, the cellulose of paper is used as the stationary phase. Capillary action is used to pull the solvents up through the paper and separate the solutes. PC is mainly an analytical technique for separating and identifying mixtures that can be colored, such as pigments. Different compounds in the sample mixture move at different rates due to differences in their solubility in the solvent and differences in their attraction to the fibers of the paper. PC takes from several minutes to several hours for development. Any substance that reacts or bonds with the paper cannot be measured using this technique. After the development, the spots corresponding to different compounds may be located by their color, or under UV light, or by treatment with iodine vapors, and so on. The paper remaining after the experiment is known as a *chromatogram*. In PC the retention factor, R_f, is a quantitative indication of how far a particular compound travels in a particular solvent.

The retention factor, R_f, is defined as $R_f = \text{D1} / \text{D2}$, where D1 is the distance the solute moves and D2 is the distance traveled by the solvent front. The components that have been separated differ in their R_f. The R_f values found can be compared to known values, and from that conclusions can be drawn. The original work in PC was carried, mainly, on Whatman no. 1 filter paper. Because of the slow separation time of the procedure (movement of mobile phase by capillary forces) during PC, the use of PC faded with the rise of TLC, which has largely replaced PC. It is, however, still a powerful teaching tool [5–7].

Thin-layer chromatography (TLC) is the chromatographic technique of applying a thin layer of an adsorbent on mainly glass or alumina plates, which is the stationary phase. This polar thin-layer cell, and the polarities of both the components of the mixture and the solvent used as the mobile phase, are the factors that determine how fast the compound under analysis travels. This kind of chromatography also uses, like PC, capillary action to move the solvent through the stationary phase. The

retention factor, R_f, plays the same important role in TLC as in PC. TLC soon became the standard analytical chromatographic method in organic and pharmaceutical laboratories, and has not yet been completely displaced by other methods mainly because of its simplicity and high speed [7–9].

Column chromatography (CC) is used to separate and purify components of a mixture. The stationary phase is a powdered adsorbent that is placed in a vertical glass column. The mixture to be analyzed is loaded on top of this column. The mobile phase is a solvent poured on top of the loaded column. The solvent flows down the column, causing the components of the mixture to distribute between the powdered adsorbent and the solvent, thus separating the components of the mixture so that as the solvent flows out of the bottom of the column, some components elute with early collections and other components elute with late fractions. CC is often used for preparative applications on scales from micrograms up to kilograms. The classical preparative chromatography column is a glass tube with a diameter from 10 to 100 mm and a height of 10 cm to 1 m, where a slurry is prepared of the eluent and the stationary-phase powder and then is poured into the column. The eluent is slowly passed through the column to advance the organic material. The individual components are retained by the stationary phase differently and separate from each other through the column with the eluent in a series of fractions [5,10].

The stationary phases are usually finely ground powders or gels and/or are microporous for an increased surface. The mobile phase or eluent is either a pure solvent or a mixture of different ones; it is chosen so that the different compounds can be separated effectively. The eluent is optimized on a small scale using TLC with the same stationary phase. New chromatographic columns have been introduced during recent years and used as the ones of biotechnology. These have been designed to permit high-flow rates at higher pressures (>15 psi). Special columns of stainless steel, high-density glass, or acrylic components have taken the place of the standard glass or plastic columns that were previously used. New column designs have allowed the development of new types of chromatography, such as high performance liquid chromatography (HPLC), which was made possible by the development of steel columns and chromatographic media that are able to handle high pressures (hundreds to thousands of psi) [10,11].

GC is the kind of chromatography in which the stationary phase is a high-boiling liquid (waxy substance or viscous oil, etc.), which is packed into a long, narrow glass, or metal column. The mixture to be analyzed is loaded by syringe in this column. The mobile phase is an inert gas that flows continuously through the column where the components of the mixture are distributed between the stationary high-boiling liquid (condensed or absorbed on it) and are moving through the column. The gaseous mixture flows through a detector at the end of the column, and if it has been successfully separated, the components show as different "peaks" on a recorder. In GC, the determining factor *Rt* shows how fast a component travels and is usually (but not always) the boiling point of the compound. If a polar high-boiling-point liquid adsorbent is used in the GC column, the polarity of the components determines the elution order [12–18].

2.4 LIQUID CHROMATOGRAPHY

The stationary phase or adsorbent in CC is solid, most commonly silica gel, alumina, and/or cellulose. Liquid chromatography can be subdivided into ion-exchange chromatography, reversed-phase (RP) chromatography, affinity chromatography, and so on [10,11].

2.4.1 Ion-Exchange Chromatography

Ion-exchange chromatography (or *ion chromatography*) is a process that allows the separation of ions and polar molecules based on the charge properties of the molecules. It can be used for almost any kind of charged molecule including proteins, nucleotides, and amino acids. The solution to be injected is usually called a *sample*, and the individually separated components are called *analytes*. It is often used in protein purification, water analysis, and quality control [10].

2.4.2 Hydrophobic Interaction Chromatography (HIC)

Not all of the common amino acids found in proteins are charged molecules. There are some amino acids that contain hydrocarbon side chains that are not charged and therefore cannot be purified by the same principles involved in ion-exchange chromatography. These hydrophobic ("water-hating") amino acids are usually buried away in the inside of the protein. Since most of the hydrophobic groups are not on the surface, the use of HIC allows a much greater selectivity than is observed for ion-exchange chromatography through the binding of these hydrophobic amino acids on a support that contains immobilized hydrophobic groups.

2.4.3 Gel-Filtration Chromatography

This technique separates proteins based on their size and shape. The support for gel-filtration chromatography is beads that contain holes, called *pores*, of certain sizes. Larger molecules, which cannot penetrate the pores, move around the beads and migrate through the spaces that separate the beads faster than the smaller molecules, which may penetrate the pores. This is the only chromatographic technique that does not involve binding of the protein to a support.

2.4.4 Affinity Chromatography

This is the most powerful technique available. It can potentially allow a one-step purification of the target molecule. In order to work, a specific ligand (a molecule that recognizes the target protein) must be immobilized on a support in such a way that allows it to bind to the target molecule. This technique has the potential to be used for the purification of any protein, provided that a specific ligand is available, such as for the purification of recombinant proteins or lectin affinity chromatography where lectins are used to separate components within the sample [19,20].

2.5 TYPES OF HPLC

High performance liquid chromatography (HPLC, also known as **high pressure liquid chromatography**) is a form of CC used in analytical chemistry as well as in biochemistry to separate, identify, and quantify compounds. HPLC utilizes a column that holds the stationary phase (chromatographic packing material), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times (R_t) of the monitored molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the eluents (solvents) chosen [4,5].

2.5.1 Normal-Phase Chromatography

Normal-phase HPLC (NP-HPLC) is a method in which analytes are separated based on their polarity. In NP-HPLC a polar stationary phase and a nonpolar mobile phase are used, and it works effectively mainly for polar analytes. The use of more polar solvents in the mobile phase will decrease the retention times of the analytes, whereas more hydrophobic solvents tend to increase retention times. NP-HPLC fell out of favor in the 1970s with the development of reversed-phase HPLC because of a lack of reproducibility of retention times [4,5].

2.5.2 Reversed-Phase Chromatography

Reversed-phase HPLC (RP-HPLC or RPC) has a nonpolar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica, which has been treated with RMe2SiCl, where R is a straight-chain alkyl group such as C18H37 or C8H17. With these stationary

phases, the retention time is longer for molecules that are less polar, while polar molecules elute more readily. The binding of the analyte to the RPC stationary phase is proportional to the contact surface area around the nonpolar segment of the analyte molecule upon association with the ligand in the aqueous eluent. The retention can be decreased by adding a less polar solvent (methanol, acetonitrile, etc.) into the mobile phase to reduce the surface tension of water. Gradient elution uses this effect by automatically changing the polarity of the mobile phase during the analysis procedure. Another important component is the influence of the pH, since this can change the hydrophobicity of the analyte. For this reason most methods use buffering agents to control the pH. Organic acids such as acetic acid or formic acid are often also added to the mobile phase. The effects of acids and buffers vary by application but generally improve the chromatographic results [4,5,10,11].

2.5.3 Size Exclusion Chromatography (SEC)

Size exclusion chromatography (SEC), also known as gel-filtration chromatography, separates particles on the basis of size. It is generally a low-resolution chromatography, and it is often reserved for the final step of a purification. It is also useful for determining the tertiary or quaternary structure of purified proteins. SEC is widely used to determine the molecular weight of polysaccharides, and it is the official technique that has been suggested by European Pharmacopoeia for the molecular-weight comparison of different commercially available low-molecular-weight heparins [4,5,10].

2.5.4 Ion-Exchange Chromatography

In ion-exchange chromatography, retention is based on the different attraction between solute ions and charged sites bound to the stationary phase. Types of ion exchangers include (i) cellulose and dextran ion exchangers (gels), which possess larger pore sizes and low charge densities, making them suitable for protein separation, (ii) polystyrene resins, which allow cross-linkage, which increases the stability of the chain, and (iii) controlled-pore glass or porous silica. This type of chromatography is widely used in water purification, ion-exchange chromatography of proteins, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides, and so on.

2.5.5 Bio-Affinity Chromatography

This chromatographic process is based on the ability of biologically active substances to form stable, specific, and reversible complexes. The formation of these complexes involves the participation of common molecular forces such as the Van der Waals interaction, electrostatic interaction, dipole–dipole interaction, hydrophobic interaction, and the hydrogen bond. An efficient, biospecific bond is formed by a concerted action of several of these forces in the complementary binding site.

2.5.6 Isocratic Flow and Gradient Elution

A separation in which the mobile-phase composition remains constant throughout the procedure is termed *isocratic* (meaning "constant composition"). A separation in which the mobile-phase composition is changed during the separation process is described as *gradient elution*.

2.6 HERBAL DRUGS

Secondary metabolites of plants comprise more than 30,000 different chemical substances that are exclusively produced by plants. The plants produce secondary metabolites as coloring, scent, or attractants; plant hormones; as well as defenses against pests. In contrast to the primary metabolites (carbohydrates, fats, proteins), secondary metabolites do not have nutrient characteristics for

humans. They are usually found in very small amounts but have a scientifically proven effect on human health as they can kill pathogenic microorganisms (bacteria and fungi), protect the immune system or the body from free radicals, and contribute to protecting humans from cancer and cardiovascular illnesses. Plants that have been intensively studied for their secondary metabolites and have shown established medical effects on humans are called *herbal medicinal products* or *herbal drugs* [21,22].

Herbal drugs can be any plant's seeds, berries, roots, leaves, bark, or flowers used by humans for medicinal purposes. Plants were already being used for medicinal purposes long before recorded history. Ancient Chinese, Egyptian, and Greek writings describe medicinal plant uses, while indigenous African and Native American cultures used herbs in their healing rituals. The Indians and Chinese developed traditional medical systems such as Ayurveda and traditional Chinese medicine, respectively, in which herbal therapies were used systematically. In the early nineteenth and twentieth centuries, when methods of chemical analysis improved and became available, scientists began extracting, isolating, and structurally determining the active ingredients of plants through modern analytical and spectral means. Later, chemists and pharmacists began the transition from raw herbs to synthetic pharmaceuticals [21–25]. The World Health Organization (WHO) has recently estimated that 80% of people worldwide rely on herbal medicines for some aspect of their primary health care while in EU countries roughly 200 plant-based medicines are available, and certain health problems such as digestive problems, dermatological problems, coughs, the common cold, migraines, premenstrual syndrome, menopausal symptoms, chronic fatigue, mild gastrointestinal disorders, anxiety, and sleeping problems can be treated with herbal drugs. Plants like *Ginkgo biloba*, *Hypericum perforatum*, *Valeriana officinalis*, *Echinacea purpurea*, and other *Echinacea* species are among the most widely used herbal medicinal plants, offering an effective treatment against several of the previously mentioned health problems [25–28].

2.6.1 Overview of Herbal Quality

For the quality control of herbal remedies, it is important to provide an overview of the types of toxic and pathogenic contaminants that may occur. These include toxic botanicals, microorganisms, microbial toxins, pesticides, fumigation agents, radioactivity, toxic metals, synthetic pharmaceuticals, and animal substances.

Assuring the quality of herbal drugs requires the standardization of crude drug materials and finished products, including the following steps, according the guidelines set by WHO as well as national and European pharmacopoeias [29,30]:

- Authentication: Botanical evaluation (stage of collection, parts of the plant collected, regional status, botanical identity like phytomorphology, microscopical and histological analysis, taxonomical identity, sensory characters, and so on). Toxic botanicals are a problem mainly due to the determination of botanical identity. The risk of *Teucrium*-induced hepatitis is a characteristic example not only for *Teucrium chamaedrys* but also for *Teucrium polium* as well [31–35].
- Foreign organic matter (herbs collected should be free from soil, insect parts, or animal excreta, etc.)
- Physiochemical character of the herbal drug: Organoleptic evaluation (sensory character: taste, appearance, odor of the drug, etc.)
- Ash values, extractive values, refractive index, polarimetric readings, and moisture content
- Volatile matter [14].
- Chromatographic and spectroscopic evaluation: TLC and HPLC methods provide qualitative and semiquantitative information about the main active constituents (active markers) present in the crude drug as chemical markers in the TLC fingerprint evaluation of herbals, according to the existing monographs in European or national pharmacopoeias [6].

- Reference to the pharmacological parameters: Biological activity profiles, bitterness values, hemolytic index, astringency, swelling factor, foaming index, etc.
- Toxicity details: Heavy metals like cadmium, lead, arsenic, mercury, etc.
- Pesticide residue: Fumigation agents. WHO and Food and Agricultural Organization (FAO) set limits on pesticides, which are usually present in the herbs. These pesticides are mixed with the herbs during the time of cultivation. Pesticides like DDT and BHC cause serious side effects in human beings. It is important that herbal products and crude herbs are controlled for the absence of unsafe levels of these substances. The European Pharmacopoeia sets general limits for pesticide residues in medicines and also has a limit for fumigation residues of ethylene oxide [29–32].
- Microbial contamination: Usually medicinal plants contain bacteria and molds are coming from soil and atmosphere. Total viable aerobic count, pathogenic bacteria like enterobacteria, *E. coli*, *Salmonella* sp., *Pseudomonous aeruginosa*, *Staphylococcus aureus*, etc. The substances known as aflatoxins will produce serious side effects if consumed along with the crude drugs. A last and unusual microbial health hazard of herbal products are that certain plant constituents are susceptible to chemical transformation by contaminating microorganisms. An example is that the molding of dried sweet clover (*Melilotus officinalis*) can result in serious hemorrhagic activity [34].
- Radioactive contamination.
- Synthetic pharmaceuticals. There is an experience of the presence of conventional pharmaceuticals as corticosteroids and nonsteroidal anti-inflammatory drugs in certain herbal medicines of Asian origin [31,32,36].
- Animal substances are also among the potential contaminants of herbal remedies. There are examples of the presence of thyroid hormones in antiobesity preparations [31,32,37], as well as of toad venom from the Chinese *Bufo* species in herbal teas, which is rich in toxic steroids and has digoxin-like activity [38].

2.6.2 Use of Fingerprinting and Marker Compounds for Identification and Standardization of Botanical Drugs

For standardization of herbal drugs, single characteristic chemical constituents, "marker compounds," may be used as potency standards in chromatographic analyses. Using well-characterized marker compounds, conventional pharmaceutical manufacturing criteria for assay and content uniformity may be applied. These marker compounds may be used to help identify herbal materials, set quality specifications for raw materials, standardize botanical preparations during all aspects of the manufacturing processes, and obtain stability profiles. HPLC analysis for marker compounds may provide additional information in the form of "chromatographic fingerprints." These marker compounds would be further analyzed into "chemical markers," which are the chemical constituents that are among the major constituents of the plant material and can easily be detected chromatographically, and "active markers," which are the chemical constituents that are proved to possess the therapeutic activity of the herbal drug [39].

2.6.3 Applications of HPLC Analyses to Herbal Drugs and Herbal Products

Chromatographic analytical methods, mainly HPLC, have been used in many different ways to determine known or unknown substances in herbal products in order to assure their quality in the industry as well as by the competent authorities. Food, beverages, perfumes, and also herbal drugs are among the herbal products that contain several naturally present secondary metabolites in their raw materials and some that form during processing [40]. HPLC is extensively used for the analysis of all these herbal products. It is also used to detect and measure contaminants from spoilage or adultering that may be harmful; this is mainly controlled by governmental agencies [41]. So, there

TABLE 2.1
The Applications and Main Uses of the Different Types of Chromatography for Herbal Drug Analyses

Type of Chromatography	Applications	Main Uses
Liquid chromatography	Pharmaceutical analyses, forensic chemistry, forensic toxicology, toxicological analyses, food chemistry, herbal drug analyses	Secondary metabolites and metal ions
Gas chromatography	Herbal drug analyses, forensic chemistry, forensic toxicology, toxicological analyses, food chemistry	Volatiles, secondary metabolites
Thin-layer chromatography	Herbal drug analyses, food chemistry	Simpler and more rapid method for checking the purity of herbal drugs (secondary metabolites, volatiles) as well as detecting pesticide or insecticide residues
Paper chromatography	Herbal drug analyses	The most common type of chromatography, used for separations of amino acids and anions, RNA fingerprinting, histamines, and antibiotics

are HPLC methods for qualitative and quantitative determination of the following: artificial colorings (Rhodamine B, etc.), aflatoxins, ochratoxin A, coumarins, genotoxic anthraquinones [42], isoflavones (known as phytoestrogens) in soy and soy products, isoflavones in red clover, pyrrolizidine alkaloids [43], caffeine, catechines (including epigallo catechin gallate in green tea), and opium and other alkaloids in various plants and mother tinctures [44]. As can be seen in Table 2.1, HPLC analyses for herbal products are the most widely used of all chromatographic techniques in the food chemistry and pharmaceutical industries, and they are also of great importance in clinical analysis [45,46] of herbal drugs (biopharmaceutics, bioequivalence, cocaine in urine, alcohol in blood, etc.) [47]; for the screening, determination, and measuring of narcotics such as opium alkaloids, cocaine, and morphine; for health consequences of acute and chronic marijuana use [47–49], and for several applications to forensic problems (forensic chemistry, forensic toxicology) [50].

2.7 CONCLUSIONS

A final aspect to be emphasized is that for every herbal product, regardless whether it is being sold as a food, food supplement, cosmetic, medical device, dietary supplement, or an approved drug, the quality of the raw material and of the finished herbal product are of great importance for consumers and for our society. In other words, assuring the safety of any herbal product requires that the quality is extensively monitored through the most convenient chromatographic methods, depending on the kind of analysis, during each step of its production, in order to achieve high safety and efficacy levels of the finished herbal products for the end consumers.

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3 Plant Products in Nutrition and Dietary Supplements: Quality Control

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CONTENTS

3.1 INTRODUCTION

For the last decades, a noticeable and ever-growing interest by millions of people worldwide in using foods containing concentrated sources of nutrients or supplemented with botanicals or botanical preparations deriving from plants have been observed. However, despite these constituents' natural origin, there is also an increasing need to establish international rules concerning their quality and safety assessment. The development and validation of a great number of comprehensive analytical methods (including HPLC and related techniques) for accurate measurement of active or potentially toxic ingredients have turned out to be crucial for the realization of this purpose.

In this paper, a short description of regulatory procedures and quality aspects relating to foods and dietary supplements in the European Community and the United States, followed by some important examples of the practical application of HPLC to the analysis of phytochemical food components, is presented.

3.2 FOODS AND DIETARY SUPPLEMENTS: CATEGORIZATION AND REGULATORY STATUS IN THE UNITED STATES AND EUROPE

The simple differentiation between foods and dietary (food) supplements is based on the fact that the former term describes products with traditional nutritional value, whereas the latter is related to products that are classified as foods or food ingredients possessing defined physiological effects, that is, able to promote health, enhance human (mental and physical) performance, and diminish risk factors of some diseases [1]. They are intended to supply nutrients (vitamins, minerals, nonsaturated fatty acids, or amino acids) that are missing or not consumed in sufficient quantity in human diets, or they can contain plant-derived constituents that may have additional health benefits. Hence, dietary supplements (sometimes also called nutraceuticals) are often placed on the borderline between food and drug substances.

In the United States, food supplements are defined according to the Dietary Supplement Health and Education Act of 1994 as the products used for dietary supplementation with the previously mentioned nutrients or botanical-sourced constituents, intended for ingestion in pill, capsule, tablet, powder, or liquid form and labeled as dietary supplements [2]. Additionally, the Food and Drug Administration (FDA) classifies these products as foods and not as drugs. Therefore, the manufacturers of dietary supplements are not required to prove their safety and efficacy as well as quality, except infant formulas and medical foods [3]. In 1995 the Office of Dietary Supplements (ODS) at the National Institutes of Health (NIH) was created. Its responsibilities comprise strengthening knowledge of dietary supplements by collecting and evaluating scientific information, including data from foreign sources; stimulating, supporting, and conducting studies on dietary supplements; and disseminating research results within the U.S. population [4].

Contrary to the FDA's standpoint, the Directive 2002/46/EC of the European Parliament and of the Council of Europe requires the confirmation of safety (both in quantity and quality) for food supplements [5]. Consequently, only those supplements that have been proved to be safe may be sold without prescription, with the additional condition that their labeling must not refer to the property of preventing, treating, or curing any human disease but only bear health claims. However, the regulatory procedures in particular European countries are different, hence, the same plant ingredients are sometimes classified as nutritional supplements or over-the-counter drugs.

3.3 QUALITY ASPECTS OF THE BOTANICAL PRODUCTS USED IN DIETARY SUPPLEMENTS

Since rapid and unrestricted expansion of both food and dietary supplements containing plant extracts or constituents have been observed in both European and U.S. markets in recent decades, the problem of establishing and harmonizing proper concepts of and qualitative criteria for these products, in order to protect consumers from any toxicological risk as well as to guarantee the positive influence of nutraceuticals on human health, still needs to be solved. Nowadays, the main problems discussed comprise the constant quality of botanical products to get a reproducible safety and efficacy profile, the strict standardization of the extracts and related good manufacturing practice (GMP) procedures, the control of genetic and toxicological safety, and a reasonable tolerability level for these products [6]. Gulati and Ottaway [7] elaborated a very clear presentation of the general quality aspects of dietary supplements containing botanical components in the European Union. The main points to consider, shown in Table 3.1, are also in accordance with the recent

TABLE 3.1
Important Quality Aspects Concerning Botanical-Sourced Dietary Supplements

1. **Botanical source**
   - Identity (scientific name, botanical family, genus, species, chemotype, common name)
   - Part of plant used
   - Contamination with other plant species
   - Chemical and microbial contamination and foreign matter (heavy metals, pesticide residues, aflatoxins, radioactivity)
2. **Growth conditions**
   - Good agricultural practices (GAP)
   - Wild or cultivated
   - Site of collection
   - Time of harvest
   - Stage of growth
   - Storage conditions postharvest
   - Pre- and postharvest treatments (use of pesticides, etc.)
3. **Raw material**
   - Specifications according to standard method (Pharmacopoeia) or validated standard method
   - Quantitative test to determine constituents relevant to their biological significance
   - Stability data
4. **Raw process applied to starting material**
   - Extraction process
   - Solvents
   - Method used
   - Specific precautions (light and temperature sensitivity)
5. **Botanical preparation**
   - Standardization criteria (markers, other relative constituents, plant/extract ratio)
   - Specifications (level/range of markers)
   - Physicochemical properties (stability data)
   - Purity criteria
   - Limits of biologically active principles
   - Level and nature of excipients
   - Formulation methodology
   - Storage conditions
6. **Specifications**
   - Quality control/assurance (methods of production, specifications)
   - Standardization to comply with existing standards based on phytochemical markers or fixed plant/extract ratio (PER); if appropriate markers are not known, chemical fingerprints (HPLC-UV, HPLC–MS) should be used.
7. **Other technical criteria**
   - Impact of production process (use of solvents, ratio of solvents)
   - Consistency of batch-to-batch analysis data
   - Fixed plant/extract ratio (PER)
   - Solvent residues
   - Pesticide residues
   - Stability data
   - Purity criteria
   - Storage conditions

guidance document on safety assessment of botanicals (whole, fragmented or cut plants, plant parts, algae, fungi, and lichens) and botanical preparations (products obtained from botanicals by various processes, for example, pressing, squeezing, extraction, fractionation, distillation, concentration, drying, and fermentation) intended for use as ingredients in food supplements. The aforementioned document was published as a draft by the European Food Safety Authority in June 2008 [8].

In the last decade, a very important initiative started to be developed by the Association of Analytical Communities (AOAC International) in collaboration with the NIH and FDA to elaborate, validate, and adopt standard assays for dietary supplements in order to monitor their quality and compare the results obtained more efficiently [9]. Because food supplements, especially those containing plant components, are very variable as regards to their chemical composition, and, in many cases, the active constituents are not known and appropriate markers must be selected, the idea of interlaboratory studies on the development of validated analytical (including chromatographic) methods of dietary supplements may lead to the elaboration of international standardized models that can be used by regulatory agencies, laboratories, and academic institutions to maintain a high level of product quality.

3.4 HPLC ANALYSIS OF PHYTOCHEMICALS IN SOME FOOD PRODUCTS

3.4.1 Application of HPLC in the Analysis of Phenolic Components in Fruit, Fruit Juices, and Wines

Plant phenolics constitute a broad range of secondary metabolites, possessing characteristic structural elements: at least one aromatic ring substituted by one or more hydroxyl groups, free or in ether, ester, or glycoside form. These compounds are identified in significant quantities in fruit, fruit juices, and wines, determining their physiological and organoleptic (taste, odor, color) properties [10]. Phenolics, in relation to aforementioned food products, comprise predominantly phenolic acids (cinnamic and benzoic acid derivatives), flavonoids, and anthocyanins (and structurally similar compounds), as well as tannins and stilbenes. Taking into account the polar character of phenolics, alcohols (methanol, ethanol, propanol) and acetone, or their combinations with water, are considered proper extraction solvents. Slightly different isolation procedures have been described for anthocyanins, where acidified organic solvents (most commonly methanol) are recommended to be used [11]. Before quantification of phenolics by HPLC, various purification and fractionation procedures need to be done to separate hydrophobic ballast compounds, such as chlorophyll, waxes, fatty acids, or terpenes. For this purpose, solid-phase extraction on ion-exchange resins, cross-linked dextranes (Sephadex LH-20), and chemically bonded phases (C_{18} silica gel) or liquid–liquid procedures have been employed, comprising classical solvent extraction or high-speed countercurrent chromatography [12].

As regards chromatographic methods, HPLC is nowadays the most widely used technique for the separation, qualitation, and quantitation of polyphenols in foods. The high efficiency of this method is mainly based on the application of reversed-phase stationary phases in combination with various detection systems, including the most advanced mass spectrometry as a very useful technique for structural analysis of phenolics [12,13]. The more important recent applications of HPLC in studies on polyphenols in fruit, fruit juices, and wines have been presented in Table 3.2.

3.4.2 HPLC Identification and Determination of Flavane Derivatives and Other Phenolics in Various Tea Cultivars

Infusions or decoctions prepared from the leaves of *Camellia sinensis* (L.) Kuntze (Theaceae), known as tea, belong to the most popular beverages consumed every day all over the world. Unfermented green tea and partially fermented pu-erh tea are especially valued by people in China and Japan, whereas fully fermented black tea is preferred in India, Africa, and Europe. This plant substance is a rich

TABLE 3.2
Recent HPLC Applications in the Analysis of Some Classes of Phenolics Contained in Fruit, Fruit Juices, and Wines

Food Sample	Phenolics	Column	Mobile Phase	Detection	Ref.
Apples, grapes	Catechins	Inertsil ODS-2 (150 × 4.6 mm ID, 5 µm) with an Opti-Guard PR C_{18} Violet A column	(A) 5% acetonitrile in 0.025 M phosphate buffer, pH 2.4; (B) 25% acetonitrile in 0.025 M phosphate buffer pH 2.4; 0–5 min, 10% B in A; 5–20 min, 10–80% B in A; 20–22 min, 80–90% B in A; 22–25 min, 90% B in A; 25–28 min, 10% B in A; 28–37 min, 10% B in A	UV, FL	[14]
Cranberry and lingonberry fruit	Catechins, flavones, flavonols, flavanones	Inertsil ODS (150 × 4 mm ID, 3 µm) with a C_{18} guard column	(A) 50 mM H_3PO_4, pH 2.5; (B) acetonitrile; *catechins*: 86% A in B; *flavonoids*: 0–5 min, 95% A in B; 5–55 min, 95–50% A in B; 55–65 min, 50% A in B; 65–67 min, 50–95% A in B	PDA, EC	[15]
Red raspberry fruit	Ellagic acid derivatives, flavonol glycosides	Lichrocart 100 RP18 column (250 × 4 mm ID, 5 µm)	(A) 5% aqueous solution of HCOOH; (B) MeOH; 0–5 min, 10–15% B in A; 5–20 min, 15–30% B in A; 20–35 min, 30–50% B in A; 35–38 min, 50–90% B in A	PDA	[16]
Red raspberry fruit	Ellagitannins, quercetin glycosides	Synergi RP-Max column (250 × 4.6 mm ID, 4 µm)	(A) 1% aqueous solution of HCOOH; (B) acetonitrile; 0–60 min, 8–21% B in A	PDA, ESI-MS	[17]
Strawberry fruit	Ellagic acid, flavonols	Prodigy ODS3 column (250 × 4.6 mm ID, 5 µm)	(A) H_2O–THF–TFA, 98:2:0.1; (B) acetonitrile; 0–2 min, 17% B in A; 2–7 min, 17–25% B in A; 7–15 min, 25–35% B in A; 15–20 min, 35–50% B in A	PDA	[18]
Nectarines, peaches, plums	Phenolic acids, flavonols, catechins, procyanidins	Nucleosil C_{18} (150 × 4.6 mm ID, 5 µm) with a guard column containing the same stationary phase	(A) 5% MeOH in H_2O; (B) 12% MeOH in H_2O; (C) 80% MeOH in H_2O; (D) MeOH; 0–5 min, 100% A; 5–10 min, 0–100% B in A; 10–13 min, 100% B; 13–35 min, 100–75% B in C; 35–50 min, 75–50% B in C; 50–52 min, 50–0% B in C; 52–57 min, 100% C; 57–60 min, 100% D	PDA, ESI-MS	[19]
Grape, cherry, raspberry, blackberry, blackcurrant juices	Anthocyanins, flavonols, stilbenoids, phenolic acids	Lichrocart RP18 column (250 × 4 mm ID, 5 µm)	(A) 5% aqueous solution of HCOOH; (B) MeOH; 0–30 min, 2–32% B in A; 30–40 min, 32–40% B in A; 40–50 min, 40–95% B in A; 50–55 min, 95% B in A	PDA	[20]

(*Continued*)

TABLE 3.2 (CONTINUED)
Recent HPLC Applications in the Analysis of Some Classes of Phenolics Contained in Fruit, Fruit Juices, and Wines

Food Sample	Phenolics	Column	Mobile Phase	Detection	Ref.
Red and white wines	Stilbenes (resveratrol derivatives), flavanonols	Prontosil C_{18} (250 × 4 mm ID, 4 μm) with a guard column containing the same stationary phase	*Stilbenes*: (A) 1% aqueous solution of TFA; (B) acetonitrile–solvent A, 80:20, v/v; 0–10 min, 15% B in A; 10–13 min, 15–18% B in A; 13–15 min, 18% B in A; 15–17 min; 18–23% B in A; 17–21 min, 23–25% B in A; 21–24 min, 25% B in A; 24–28 min, 25–32% B in A; 28–30 min, 32% B in A; 30–33 min, 32–40% B in A; 33–38 min, 40% B in A; 38–44 min, 40–70% B in A; 44–47 min, 70–80% B in A; 47–52 min, 80–90% B in A; 52–53 min, 90–100% B in A; 53–58 min, 100% B; *flavanonols*: the same solvents (A and B); 0–10 min, 18% B in A; 10–17 min, 18–23% B in A; 17–21 min, 23–24.5% B in A; 21–27 min, 24.5–31.5% B in A; 27–30 min, 31.5–40% B in A; 30–32 min, 40–80% B in A; 32–35 min, 80–100% B in A; 35–40 min, 100% B	UV, FL	[21]
Grape, red wine	Catechins, procyanidins	Superspher 100 RP18 column (250 × 4 mm ID, 4 μm)	(A) H_2O; (B) 10% aqueous solution of CH_3COOH; *catechins*: 0–5 min, 10–80% B in A; 5–29 min, 80–100% B in A; 29–45 min, 100% B; *procyanidins*: 0–40 min, 10–70% B in A; 40–55 min, 70–85% B in A; 55–74 min, 85–100% B	PDA	[22]
Red wine	Catechins, procyanidins	Nucleosil 120 column (125 × 4 mm ID, 4 μm)	(A) 2% aqueous solution of HCOOH; (B) acetonitrile–H_2O–HCOOH, 80:18:2, v/v/v; 0–15 min, 15–75% B in A; 15–20 min, 75–100% B in A	ESI-MS	[23]
Red wine	Anthocyanins	Ultrasphere ODS column (250 × 4.6 mm ID, 5 μm)	(A) 10% aqueous solution of HCOOH; (B) acetonitrile–H_2O–HCOOH, 3:6:1, v/v/v; 0–70 min, 20–85% B in A; 70–75 min, 85–100% B in A; 75–85 min, 100% B	PDA	[24]

Red wine	Anthocyanins	*LC-PDA*: Luna C_{18} column (250 × 4.6 mm ID, 5 µm); *LC-MS*: Luna C_{18} column (250 × 2 mm ID, 3 µm)	(A) 7.5% aqueous solution of HCOOH; (B) 7.5% HCOOH in acetonitrile; 0–1 min, 3% B in A; 1–11 min, 3–15% B in A; 11–23 min, 15–25% B in A; 23–27 min, 25–30% B in A; 27–31 min, 30% B in A	PDA, ESI-MS	[25]
Red wine	Anthocyanins	Superspher 100 RP18 column (250 × 4.6 mm ID, 5 µm)	(A) 10% aqueous solution of HCOOH; (B) MeOH–H_2O–HCOOH, 45:45:10, v/v/v; 0–20 min, 35–95% B in A; 20–25 min, 95–100% B; 25–30 min, 100% B	UV, APCI-MS	[26]
Red wine	Anthocyanin-derived pigments	Aqua C_{18} column (150 × 4.6 mm ID, 5 µm)	(A) 0.1% aqueous solution of TFA; (B) acetonitrile; 0–5 min, 10% B in A; 5–20 min, 10–15% B in A; 20–25 min. 15% B in A; 25–30 min, 15–18% B in A; 30–50 min, 18–35% B in A	PDA, ESI-MS	[27]
Red wine	Pyranoanthocyanin-flavanol pigments	*LC-PDA*: C_{18} ODS column (250 × 4.6 mm ID, 5 µm); *LC-MS*: Aqua C_{18} column (150 × 4.6 mm ID, 5 µm)	*LC-PDA*: (A) 10% aqueous solution of HCOOH; (B) acetonitrile–H_2O–HCOOH, 8:1:1; 0–70 min, 15–35% B in A; 70–75 min, 35–80% B in A; 75–90 min, 80% B in A; 90–100 min, 80–15% B in A; *LC-MS*: (A) 0.1% aqueous solution of TFA; (B) acetonitrile; 0–5 min, 10% B in A; 5–20 min, 10–15% B in A; 20–25 min, 15% B in A; 25–30 min, 15–18% B in A; 30–50 min, 18–35% B in A	PDA, ESI-MS	[28]
Red wine	Pyranoanthocyanins	*C_{18} columns*: Lichrocart Purospher RP-18e (250 mm × 4 mm ID, 5 µm), YMC-Pack ODS-AM (250 × 4.6 mm ID, 5 µm), Kromasil 100 (250 × 4.6 mm ID, 5 µm); *C_{12} columns*: Synergi max RP 80A (250 × 4.6 mm ID, 4 µm)	(A) H_2O–HCOOH–acetonitrile, 87:10:3, v/v/v; (B) H_2O–HCOOH–acetonitrile, 40:10:50, v/v/v; 0–15 min, 6–30% B in A; 15–30 min, 30–50%; 30–35 min, 50–60% B in A, 35–41 min, 6% B in A	PDA	[29]

Note: APCI-MS, atmospheric pressure chemical ionization–mass spectrometry; EC, electrochemical; ESI-MS, electrospray ionization–mass spectrometry; FL, fluorescence; MeOH, methanol; PDA, photodiode-array; THF, tetrahydrofuran; TFA, trifluoroacetic acid.

source of dietary polyphenols, including flavane derivatives (catechins, theaflavins), and phenolic acids (chlorogenic, caffeic, gallic). In this group, catechins are the most abundant ingredients in fresh tea leaves, affecting the color, flavor, and bitter, astrigent taste of manufactured tea. Polyphenolic compounds (mainly catechins) are also reported to have beneficial protective effects against human degenerative diseases, such as atherosclerosis [30], chronic liver failure [31], or some kinds of cancer [32–35]. The second group of biologically active tea constituents are purine (methylxanthine) alkaloids: caffeine, theophylline, and theobromine, which stimulate the central nervous system and may improve alertness and reaction time and mitigate tiredness.

Due to its importance in the food and pharmaceutical industry, the assessment of tea quality using routine control methods has recently become very essential. Several analytical methods have been reported for the qualification and quantification of phenolics, their polymerization products, and/or methylxanthines. In order to isolate the aforementioned constituents from tea leaves, a wide variety of extraction conditions have been proposed, including the use of water at 80°C [36–38]; water at 90°C [39]; boiling water in short (3–5 min) extraction procedures [40–41]; 80% methanol for 3 h, followed by the same solvent containing 0.15% HCl for 3 h [42]; 80% ethanol at 60°C [43]; ethanol at room temperature [44]; 80% acetone for 2 weeks [45]; or 50% (v/v) aqueous acetonitrile [46]. A new idea in the process of isolation of tea catechins was the employment of a very efficient technique—pressurized liquid extraction—that under optimum conditions, comprising the use of methanol as an extraction solvent at a temperature of 160–180°C, guaranteed high recoveries of these constituents [47].

The method of choice for the analysis of tea polyphenols has traditionally been high performance liquid chromatography (HPLC). Systematic studies done by Dalluge and coworkers [36] on six reversed-phase columns—(A) Zorbax Eclipse XDB-C_{18}, (B) Zorbax Rx-C_{18}, (C) PAH Hypersil, (D) SMT OD-5-100, (E) Phenomenax Ultracarb 5 ODS, and (F) Zorbax ODS-C_{18}—documented that the use of deactivated monomeric C_{18} stationary phases and a gradient elution system with acid-containing buffers are necessary for the efficient separation and quantification of green tea catechins and caffeine (Figure 3.1). In U.S. laboratories, a simple and fast HPLC method has been developed for simultaneous determination of catechins, caffeine, and gallic acid in green, oolong, black, and pu-erh teas with photodiode array (PDA) detection. After multiple extraction procedures with aqueous methanol and acidic methanol solutions, tea extracts were separated within 20 min on a C_{18} column using a gradient elution with varying proportions of solvent A (water–acetic acid, 97:3, v/v) to solvent B (methanol). The results obtained indicated that unfermented green teas possessed higher catechin content but lower levels of gallic acid than fully fermented oolong, black, and pu-erh teas [42]. A study done by scientists from Taiwan [48] had a similar scope, dealing with effects of different steeping methods and storage on caffeine, catechin, and gallic acid content in various bag tea infusions. The HPLC method with PDA detection, a Luna C_{18} column and a step-gradient solvent system consisting of acetonitrile and 0.9% aqueous acetic acid were used for the analysis. The authors documented that for all kinds of teas (black, green, oolong, paochoung, and pu-erh) the second tea infusion contained the highest concentration of caffeine, catechins, and gallic acid, when bags were steeped in 70°C water, while the first infusion was the richest in these constituents when higher extraction temperatures (85°C and 100°C) had been used.

Enzyme-catalyzed fermentation of green tea to red and black teas results in oxidation and polymerization of the catechins to more complicated polyphenolic compounds—theaflavins and thearubigins possessing additional anticarcinogenic and health-protective activities. Within 27 min, theaflavins have been separated by HPLC together with six other tea ingredients such as caffeine, adenine, theophylline, quercetin, gallic acid, and caffeic acid, using an analytical cartridge system (Partisphere 5 C_{18}, 5 μm, 110 × 4.6 mm ID) and gradient elution with 5% (v/v) acetonitrile containing 0.035% (v/v) trifluoroacetic acid (solvent A) and 50% (v/v) acetonitrile with 0.025% (v/v) trifluoroacetic acid (solvent B) [39]. The gradient profile started at 10% B in A, increased to 20% B in A at 10 min, to 40% at 16 min, to 50% at 20 min, and back to 40% B in A from 25 to 27 min (Figure 3.2).

In the last decade, because of the complexity of the polyphenolic fractions occurring in various tea samples subjected to the manufacturing (fermentation) process, apart from classical UV, other

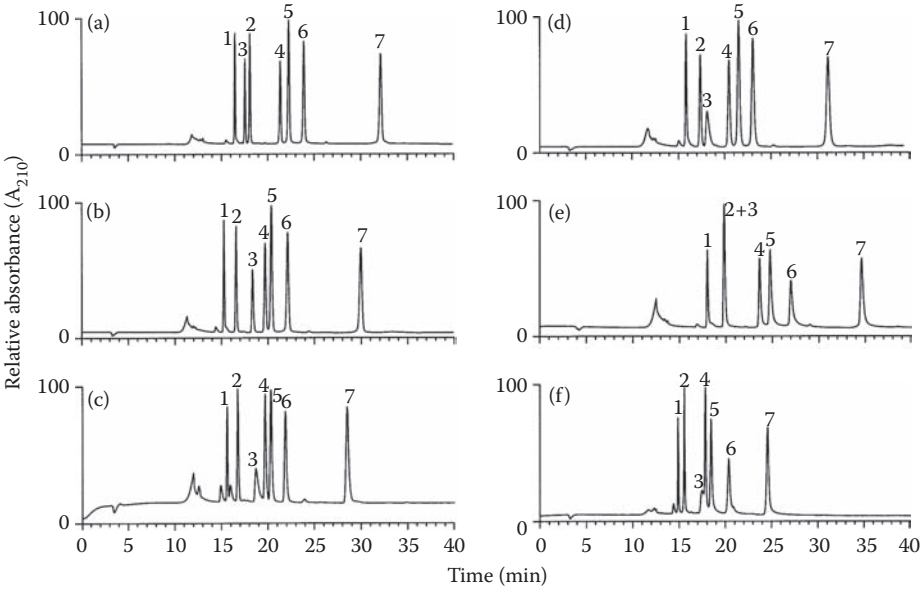


FIGURE 3.1 Comparison of six reversed-phase HPLC columns for the chromatographic separation of six catechins and caffeine in a standard mixture. For a description of each column A–F see the text. Peak identification: (1) epigallocatechin; (2) (+)-catechin; (3) caffeine; (4) epicatechin; (5) epigallocatechin gallate; (6) gallocatechin gallate; and (7) epicatechin gallate. (From Dalluge, J.J., Nelson, B.C., Brown, T.J., and Sander, L.C., *J. Chromatogr. A*, 793, 269, 1998. With permission.)

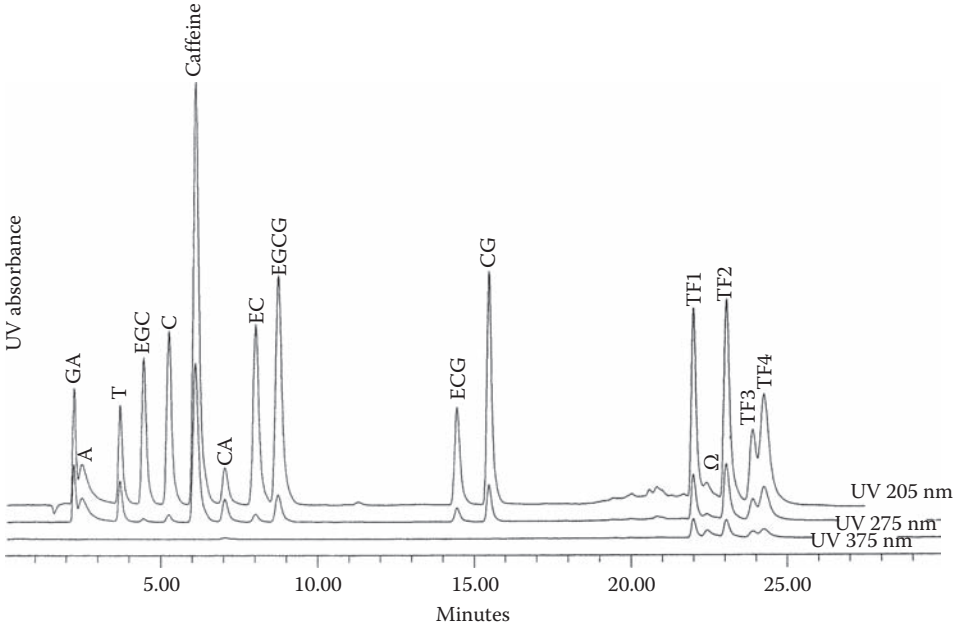


FIGURE 3.2 Chromatograms of a standard mixture containing (+)-catechin (C), catechin gallate (CG), (–)-epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), epigallocatechin-3-gallate (EGCG), caffeine, adenine (A), theophylline (T), gallic acid (GA), caffeic acid (CA), and theaflavins (TF1–TF4) monitored at UV 205, 275, and 375 nm. (From Lee, B.L. and Ong, C.N., *J. Chromatogr. A*, 881, 442, 2000. With permission.)

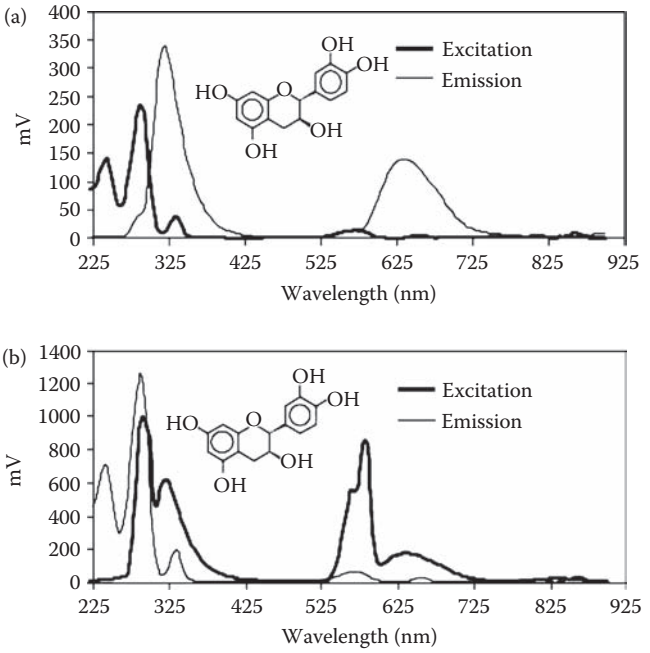


FIGURE 3.3 Fluorescence excitation and emission spectra for (a) catechin and (b) epicatechin. (From Piñeiro, Z., Palma, M., and Barroso, C.G., *J. Chromatogr. A*, 1026, 20, 2004. With permission.)

detection techniques have been employed. For example, HPLC analysis of tea catechins (Figure 3.3) was performed using fluorescence detection with optimized excitation (290 nm) and emission (320 nm) wavelengths [47]. Some new studies have utilized HPLC combined with atmospheric pressure chemical ionization–mass spectrometry (APCI-MS) for separation of 12 catechins in green and black tea infusions [49], as well as coupled with electrospray ionization–mass spectrometry (ESI-MS) for analysis of theanine, chlorogenic acid, purine alkaloids, and catechins [50]; catechins and theaflavins [51]; and theaflavins, including their methylated forms [52].

3.4.3 HPLC of Bitter and Estrogenic Phenolic Components of Hop Resins and Beer

Beer is one of the oldest man-made fermented aqueous drinks, starting to be produced probably three thousand years B.C.E. For several centuries, hops, being the female flower cones of the hop plant (*Humulus lupulus* L., Cannabaceae), have been an integral part of beer brewing, providing this beverage with important organoleptic properties (aroma, bitterness) and stability. The hop cones contain lupulin glands with hard and soft resins. The latter are rich in bitter phloroglucinol derivatives: α- and β-acids (also known as humulones and lupulones). Alpha acids, during the brewing process, undergo thermal isomerization to intensively bitter compounds—isohumulones that give beer its specific taste, exhibit bacteriostatic properties, and play an essential role in enhancing the stability of beer foam [53]. Besides bitter acids, another group of polyphenolic compounds identified in hops are prenylflavonoids; the most important chalcone—xanthohumol,which under specific conditions (increased temperature and pH value) may be converted to isoxanthohumol—is the main prenylflavonoid present in beer [54]. All the aforementioned compounds determine the quality and physiopharmacological properties of this beverage, comprising antibacterial, antifungal, and stomachic effects together with mild sedative activity. In recent years some prenylated chalcones (in particular, xanthohumol) have been shown to exert cancer chemopreventive activity, while 8-prenylnaringenin has been characterized as one of the most potent phytoestrogens isolated until now [55].

Because of the lipophilic nature of hop constituents, a wide range of organic solvents have been used for their extraction, including alcohols, chloroform, acetone, and hexane [56]. In the last three decades, novel and very effective preparative techniques have also been developed for isolation of phytochemicals from hop cones. One of them is supercritical fluid extraction (SFE), with carbon dioxide as a nonpolar solvent, giving very high recoveries of the most valuable hop constituents: humulones, prenylflavonoids, and essential oils, which influence better beer aroma and flavor [57]. Langezaal and coworkers [58] obtained the highest yield of both bitter and volatile hop constituents using SFE under optimized conditions, comprising an extraction temperature of 40°C and a pressure of 20 MPa. In another study, supercritical CO_2 (with 80% aqueous ethanol added as a modifier) has been employed for isolation of prenylflavonoids from hops, using optimum conditions: 50°C, 25 MPa, and with the ratio of solvent to material equal to 50% [59]. One modern method used for preparative separation of α- and β-bitter acids from a crude supercritical CO_2 extract of hop cones was centrifugal partition chromatography. After elution of α-acids, performed in the system toluene–0.1 *M* triethanolamine–HCl, pH 8.4, the aqueous mobile phase was changed to 0.2 *M* diethanolamine in water–methanol (4:1, v/v), adjusted to pH 9.75 with phosphoric acid (85%), and saturated with toluene. The six main bitter acids (cohumulone, humulone, adhumulone, colupulone, lupulone, and adlupulone) have been obtained using this technique [60].

Despite the hydrophobic character of hop resin constituents, reversed-phase HPLC (RP-HPLC) with UV detection has been employed as the predominant technique for the qualitative and quantitative analysis of hop cones and beer components, with isocratic or gradient eluent modes [61,62]. However, in the early 1990s, problems related to the interaction of bitter acids with trace metals present in stationary phases were reported, resulting in poor resolution and quantitation [63,64]. Besides, the instability and structural similarity of hop bitter acids caused difficulties in routine analyses. The sensitivity and selectivity of UV detection turned out to be insufficient for direct identification of these compounds, especially as regards the more uncommon hop acids in complex mixtures. Additionally, the process of isomerization of the hop α-acids to iso-α-acids or their further reduction to dihydroiso-α-acids (dihydroisohumulones) or tetrahydroiso-α-acids (tetrahydroisohumulones), used in the brewing process to enhance both the light and the foam stability of beer, significantly complicated the proper identification of all its constituents [65]. Progress in the chromatographic separation and qualitation of hop acids started to be noticed when volatile mobile-phase additives were introduced to enable HPLC hyphenation to MS detection. Using ammonium acetate or acetic acid in the mobile phase and ESI in the negative mode, hop acids were analyzed in beer after direct injection. Humulones (α-acids) were found to be present in this beverage at concentration levels of 150–200 μg/L, while lupulones (β-acids) were absent [66]. The HPLC method of simultaneous analysis of iso-α-acids and reduced iso-α-acids in beer (without sample preconcentration) has been also elaborated with both UV and MS detection working in APCI mode [65]. Zhang and coworkers [67] applied the on-line coupling of RP-HPLC to APCI tandem mass spectrometry (APCI-MS/MS) for the separation and identification of hop acids in the crude extract of *Humulus lupulus*. The solvent system consisting of acetonitrile–aqueous formic acid was used to give the proper separation of six main hop bitter acids within 30 min. The application of selected reaction monitoring (SRM), with a group of qualitatively relevant selected precursor–product ion transitions for each bitter acid in a single HPLC run, led to the identification of minor acidic compounds belonging to this group (Figure 3.4). Another hyphenated method used for the precise determination of the chemical structure of separated hop and beer bitter acids was the on-line coupling of HPLC and nuclear magnetic resonance (NMR) [68]. The study documented the high usefulness of HPLC-NMR stopped-flow measurements in the qualitative analysis of various hop bitter acids, including humulones, isohumulones, dihydroisohumulones, and tetrahydroisohumulones (Figure 3.5). The unique result of the performed experiment was the possibility of identifying not only all hop constituents but also their stereoisomeric forms.

Prenylflavonoids represent the second class of phytochemicals occurring in both the inflorescences of the hop plant and beer that have been qualified and quantified by means of the HPLC

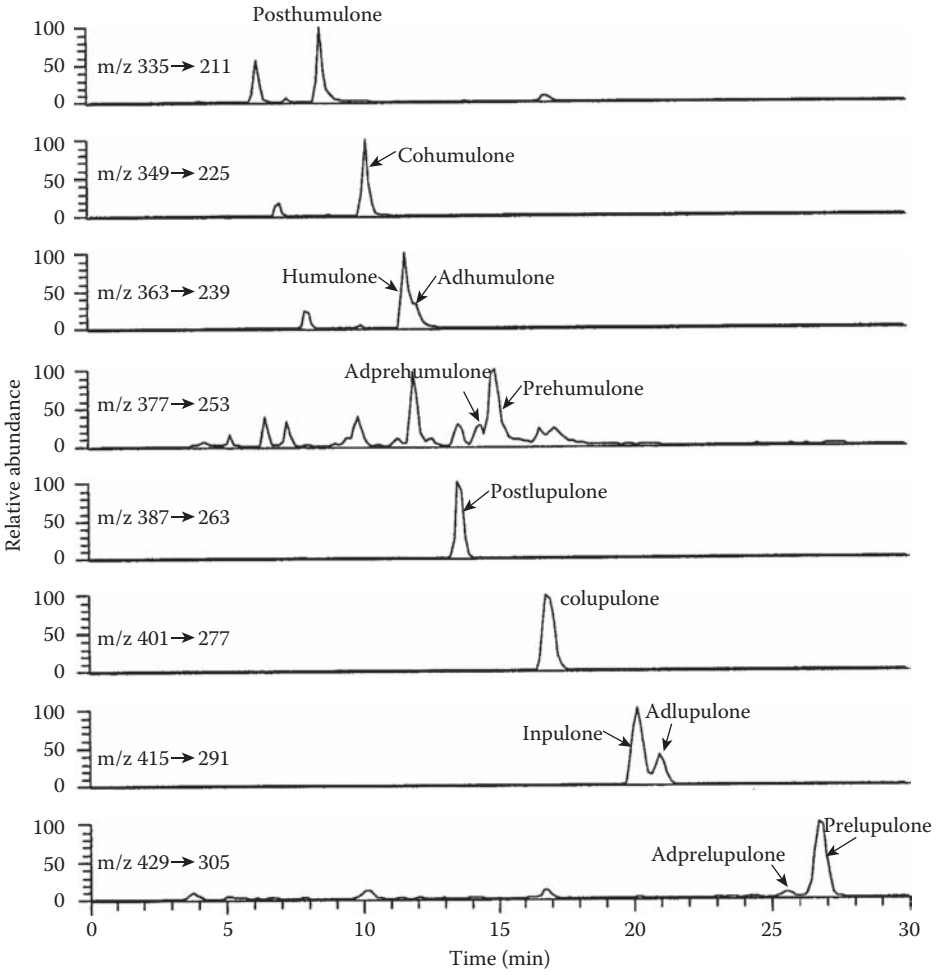


FIGURE 3.4 Selected reaction monitoring–liquid chromatography–tandem mass spectrometry (SRM-LC-MS/MS) chromatograms of hop bitter acids from the crude extract. (From Zhang, X., Liang, X., Xiao, H., and Xu, Q., *J. Am. Soc. Mass Spectrom*., 15, 184, 2004. With permission.)

technique. These compounds are generally found in beer as flavanones, formed by isomerization of their corresponding chalcones during boiling. The most abundant prenylflavanone of this beverage is isoxanthohumol, followed by 6-prenylnaringenin and 8-prenylnaringenin [69]. The latter compound has recently received a great deal of interest as a potent phytoestrogen showing significant binding affinity for two human estrogen receptors, ERα and ERβ [55]. A sensitive, selective, and robust HPLC method coupled with electrospray ionization has been developed for systematic investigation of 8-prenylnaringenin in hop varieties, hop products, and beers. The separation of hop and beer constituents was achieved on a reversed-phase C_{18} column with a linear solvent gradient comprised of formic acid in water (solvent A) and acetonitrile (solvent B) to cause stronger retention of polyphenols and to improve their resolution and symmetry of peak shapes and to facilitate ionization of hop flavonoids [70]. Stevens and coworkers [71] developed a method for quantitation of six prenylflavonoids (xanthohumol, isoxanthohumol, desmethylxanthohumol, 6- and 8-prenylnaringenins, and 6-geranylnaringenin) in hops and beer by HPLC-MS/MS. A triple quadrupole mass spectrometer, equipped with a heated nebulizer-APCI interface, was used. Isoxanthohumol, formed by isomerization of xanthohumol during the brewing process, was the most abundant flavonoid in 13 commercial beers tested, ranging from 0.04 to 3.44 mg/L.

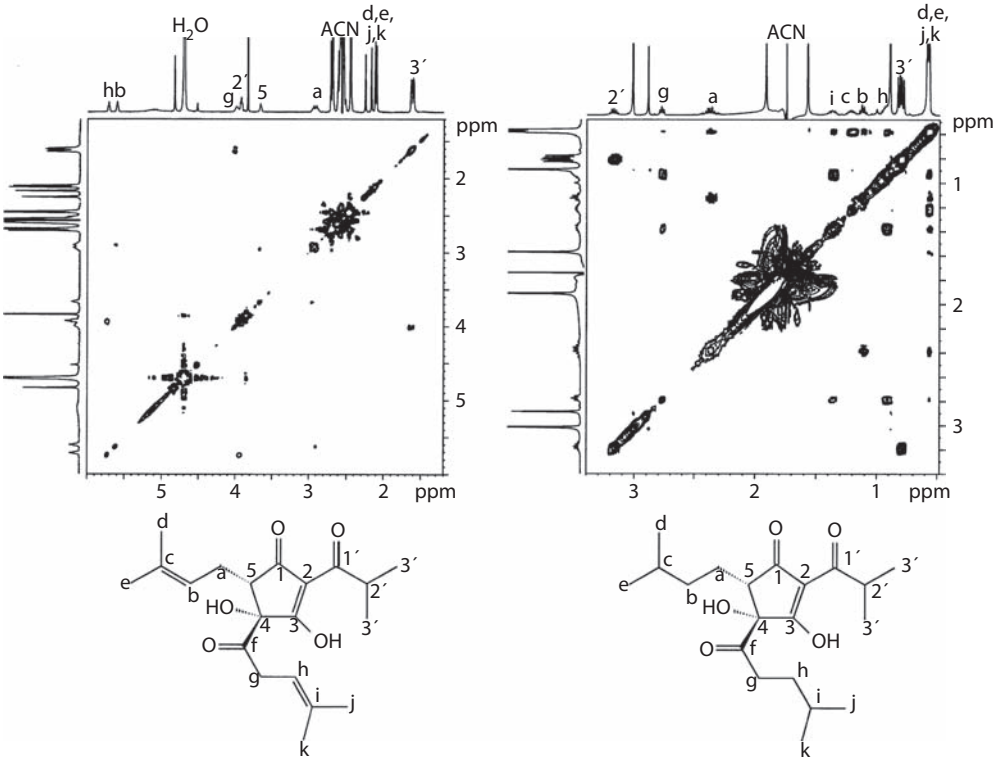


FIGURE 3.5 Two-dimensional nuclear magnetic resonance (2D NMR) stopped-flow spectra of H,H COSY spectrum of cis-iso-co-humulone (left); TOCSY spectrum of *cis*-tetrahydroiso-co-humulone (right). (From Pusecker, K., Albert, K., and Bayer, E., *J. Chromatogr. A*, 836, 251, 1999. With permission.)

Because of the antioxidant properties of both hop bitter acids and prenylflavonoids, an electrochemical detector (ECD) with coulometric detection has been used for the quantification of xanthohumol together with α- and β-acids, and the results have been compared with those obtained by UV detection. The separation of compounds was performed on a C_{18} column using isocratic elution with methanol–50 mM potassium phosphate–*ortho*-phosphoric acid (80:20:0.25, v/v/v). The ECD method turned out to be very sensitive, as the detection limits of analytes were at least 8.8–24 times lower than those reported for the UV detector [72].

3.4.4 Application of HPLC to the Analysis of Carotenoids in Foods

Carotenoids represent a group of the most widespread natural pigments, occurring in plant cell chromoplasts and determining the color (from yellow to orange and red to even almost black) of numerous fruits and vegetables. Taking into account their content in plant-derived foods, the most predominant compounds are β-carotene, β-cryptoxanthin, lycopene, rubixanthin, zeaxanthin, lutein, and violaxanthin. The nutritional and physiopharmacological importance of carotenoids comes mainly from the provitamin A activity of β-carotene and related compounds as well as from their strong antioxidant potential, as they are involved in the scavenging of reactive oxygen species (ROS) that cause oxidative damage to biomolecules [73]. For these reasons, carotenoids are known to prevent some degenerative diseases, such as age-related macular degeneration (AMD) [74], atheromatosis, osteoporosis, diabetes, cardiovascular disease, and cancer [75–77].

In the last decade, because of these health and nutritional properties, strong emphasis was put on obtaining accurate data on the content and qualitative profile of carotenoids. However, because

of the high diversity and the presence of conjugated double bonds in a great number of carotenoid molecules, which influence the compounds' instability in the presence of light, temperature, and acidic or oxygen agents, proper sample-preparation procedures are still required. Traditionally, several techniques have been employed, including the simplest one—organic solvent extraction. Carotenoids contained in fruits or vegetables are often extracted from the homogeneous samples with acetone, until no color is observed to remain in the plant tissue. Thompson et al. [78] used a mixture of hexane, acetone, and ethanol (50:25:25, v/v/v), followed by treatment with diethyl ether in a separating funnel and 10% aqueous NaCl solution to enable the transfer of lycopene from tomatoes to the nonpolar phase. The water layer was then discarded, and the organic phase was treated with Na_2SO_4 to remove aqueous residue.

As the majority of carotenoids identified in vegetables exist in the form esterified by fatty acids, to simplify their further chromatographic separation, the saponification of extract samples is needed [79,80]. For this purpose, organic fractions containing carotenoid esters are saponified with 10% methanolic KOH solution (w/v) and left for some time but mixed periodically. Then, after adding water, the carotenoids released after hydrolysis are transferred (by shaking in a separatory funnel) to diethyl ether, and the organic layer is washed with water until neutrality is reached. Last, the aqueous layer is removed, and the organic phase is passed through the bed of anhydrous Na_2SO_4 to dry the obtained pigments [81]. A modern alternative to solvent extraction is countercurrent chromatography, which in fact is an automated version of liquid–liquid extraction, allowing noninvasive isolation of labile carotenoid compounds. This technique has been successfully applied to the isolation of carotenoids from *Gardenia* [82]. As regards other mild, nondestructive methods using compressed fluids as extracting agents, supercritical CO_2 extraction (SC-CO_2) should be included in this group. A very efficient SC-CO_2 of carotenoids from carrot, using canola oil as a cosolvent, has been elaborated by Sun and Temelli [83]. The cosolvent addition improved α- and β-carotene yields more than two times and lutein yields more than four times compared to results obtained with SC-CO_2 alone. Recently, SC-CO_2 has also been effectively used for the isolation of carotenoids from pumpkins [84], rosehips [85], and pitanga fruits [86].

Purified and concentrated extracts containing both free and esterified carotenoid compounds isolated from foods are predominantly analyzed using a wide variety of HPLC methods. These are presented in Table 3.3, taking into account the latest published papers concerning the determination and quantification of carotenoids in various food samples.

To guarantee the effective separation and identification of carotenoids, gradient elution, normal- (silica, alumina) and reversed-phase stationary phases, together with PDA and MS detection techniques have been employed [98]. A special achievement that led to a significant improvement in the HPLC separation of carotenoid compounds was the introduction of polymeric C_{30} liquid chromatographic columns by Sander and coworkers [99]. As these authors documented, with the classical monomeric C_{18} columns, lutein and zeaxanthin were not separated, and other nonpolar carotenoid isomers were poorly resolved. Better separation of the hydrocarbon carotenoids was achieved with the polymeric C_{18} columns; however, *cis/trans* isomers of β-carotene were still unresolved. The situation changed when C_{30} columns were introduced, as these permitted improved separation of complex mixtures containing both polar and nonpolar carotenoid isomers.

In 2004, Pól and coworkers [88] published a highly innovative method for the analysis of thermally unstable carotenoids (lycopene) in fruit and vegetable extracts. SFE with carbon dioxide as the extraction medium was coupled on-line to HPLC. The CO_2 outlet from the SFE device was connected to a monolithic Chromolith RP-18e column, which was placed in a thermostated chamber and employed both for trapping and for analyte separation (Figure 3.6). Thanks to the on-line construction of SFE-HPLC apparatus, lycopene was not exposed to destructive conditions (air, light), and the possibility of obtaining inaccurate analytical results was thereby eliminated.

In 2008, Dugo et al. [100] elaborated and presented a novel strategy comprising different (normal- and reversed-phase), orthogonal two-dimensional HPLC methods, which have been effectively

TABLE 3.3
Selected New HPLC Applications in Analysis of Carotenoids Occurring in Food Samples

Food Sample	Carotenoids	Column	Mobile Phase	Detection	Ref.
Rosehip fruit	Lycopene, β-carotene, lutein	5C18-MS Waters (150 × 4.6 mm ID, 5 μm); column temperature: 30°C	(A) acetonitrile; (B) 2-propanol; (C) MeOH; (D) H_2O; 39:52:5:4 A/B/C/D; flow rate: 1.4 mL min^{-1}	Vis (450 nm)	[85]
Pitanga fruit	Lycopene, rubixanthin, β-cryptoxanthin	C_{18} Nova-Pak (300 × 3.9 mm ID, 4 μm); column temperature: 29°C	(A) acetonitrile; (B) H_2O; (C) EtOAc; 0 min, 88:10:2 A/B/C; 15 min, 85:0:15 A/B/C; 45 min, 85:0:15 A/B/C; flow rate: 1 mL min^{-1}	PDA, APCI-MS	[86]
Carrot	α-Carotene, β-carotene, lutein	Supelcosil LC-18 (150 × 4.6 mm ID, 5 μm) column	(A) MeOH; (B) acetonitrile; 90:10 A/B; flow rate: 1 mL min^{-1}	PDA (450 nm)	[83]
Asian pumpkin fruit	β-Carotene, lutein, lycopene, α-carotene	Prodigy C_{18} ODS3 (250 × 4.6 mm ID, 5 μm) column	(A) acetonitrile–THF–MeOH–1% aqueous $(NH_4)_2SO_4$ (85:5:5:5, v/v/v/v); (B) acetonitrile–THF–MeOH–1% aqueous $(NH_4)_2SO_4$ (55:35:5:5, v/v/v/v); 0–10 min, 5% B in A; 10–29 min, 5–95% B in A; 29–35.9 min, 95% B in A; 35.9–36 min, 95–60% B in A; 36–44.9 min, 60% B in A; 44.9–45 min, 60–5% B in A; 45–48 min, 5% B in A; flow rate: 1 mL min^{-1}	PDA (452 nm)	[84]
Vegetable oil, fruit juices, wheat bran	β-Carotene, β-cryptoxanthin, lutein, zeaxanthin	Zorbax Rx-SIL (150 × 2.1 mm ID, 5 μm)	(A) hexane; (B) 1% isopropanol in EtOAc (v/v); 0–5 min, 1–10% B in A; 5–20 min, 10–50% B in A; flow rate: 0.5 mL min^{-1}	APCI-MS/MS, ESI-MS/MS	[87]
Tomato,ruby grapefruit, pomelo red grapefruit, watermelon, papaya, tomato ketchup and paste, rosehip paste	Lycopene	Chromolith, RP-18e (300 × 4.6 mm ID) monolithic column	(A) acetonitrile; (B) *t*-BME; 90:10 A/B; flow rate: 1 mL min^{-1}	PDA	[88]
Mango fruit	*Trans* and *cis* isomers of β-carotene, violaxanthin and its *cis* isomers, neochrome, luteoxanthin, neoxanthin and its *cis* isomers, zeaxanthin, *cis*-lutein	YMC C_{30} (250 × 4.6 mm ID, 5 μm) column	(A) MeOH–iPrOH (99:1, v/v); (B) CH_2Cl_2; 0–15 min, 100% A; 15–45 min, 70% A in B; 45–60 min, 70% A in B; 60–65 min, 70–100% A; flow rate: 1 mL min^{-1}	PDA (450 nm)	[89]

(*Continued*)

TABLE 3.3 (CONTINUED)
Selected New HPLC Applications in Analysis of Carotenoids Occurring in Food Samples

Food Sample	Carotenoids	Column	Mobile Phase	Detection	Ref.
Mango fruit	Violaxanthin, violaxanthin dibutyrate, β-carotene	YMC C_{30} (250 × 4.6 mm ID, 5 μm) column, including a C_{30} (10 × 4 mm ID, 5 μm) guard column kept at 35°C	(A) *t*-BME–MeOH–H_2O (90:6:4, v/v/v/); (B) *t*-BME–MeOH–H_2O (15:81:4, v/v/v/); 0–39 min, 1–56% B in A; 39–45 min, 56–100% B; 45–50 min, 100–1% B in A; 50–55 min, 1% B in A; flow rate: 1 mL min^{-1}	APCI-MS	[90]
Tomato juice	Lycopene, β-carotene, lutein	YMC C_{30} (250 × 4.6 mm ID, 5 μm) column	(A) acetonitrile–BuOH (7:3, v/v); (B) CH_2Cl_2; 0–20 min, 1–4% B in A; 20–50 min, 4–10% B in A; 50–55 min, 10–1% B in A; flow rate: 2 mL min^{-1}	PDA (476 nm)	[91]
Lettuce, broccoli, mango, red pepper, yellow pepper, green pepper, carrot, rutabaga, sweet potato	β-Carotene, α-carotene, lycopene, capsanthin, lutein, β-cryptoxanthin, phytoene	YMC C_{30} (250 × 4.6 mm ID, 5 μm) column, coupled to a C_{30} (20 × 4.6 mm ID, 5 μm) guard column kept at 25°C	(A) MeOH; (B) 20% aqueous MeOH, containing 0.2% ammonium acetate; (C) *t*-BME; 0–12 min, 95:5 A/B; at 12 min, 80:5:15 A/B/C; a linear gradient to 30:5:65 A/B/C at 30 min; flow rate: 1 mL min^{-1}	PDA	[92]
Potatoes	Violaxanthin, antheraxanthin, lutein, zeaxanthin, neoxanthin, β-cryptoxanthin, β-carotene, and carotenoid esters	YMC C_{30} (250 × 4.6 mm ID, 5 μm) column, equipped with a Nucleosil C_{18} (10 × 4.6 mm ID, 5 μm) precolumn kept at 35°C	*System 1 (for HPLC)*: (A) MeOH–H_2O–triethylamine (90:10:0.1, v/v/v); (B) MeOH–*t*-BME–H_2O–triethylamine (6:90:4:0.1, v/v/v/v); 0–8 min, 99% A in B; 8–45 min, 99–0% A in B; 45–50 min, 0–99% A in B; 50–55 min, 99% A in B; flow rate: 1 mL min^{-1}; *System 2 (for LC–APCI–MS)*: (A) MeOH–*t*-BME–H_2O–triethylamine (81:15:4:0.1, v/v/v/v); (B) MeOH–*t*-BME–H_2O–triethylamine (6:90:4:0.1, v/v/v/v); 0–39 min, 99–44% A in B; 39–45 min, 44–0% A in B; 45–50 min, 0–99% A in B; 50–55 min, 99% A in B; flow rate: 1 mL min^{-1}	PDA (450 nm); APCI-MS	[93]

Red pepper fruit	Lutein, β-carotene, neoxanthin, violaxanthin, luteoxanthin, α- and β-cryptoxanthin, α-carotene	Chromsyl C_{18} (250 × 4.6 mm ID, 6 μm) column	*System 1*: (A) 12% H_2O in MeOH; (B) MeOH; (C) 30% CH_2Cl_2 in MeOH; *System 2*: (A) 10% H_2O in MeOH; (B) MeOH; (C) 30% CH_2Cl_2 in MeOH; 0–2 min, 100% A; 2–10 min, 100–80% A in B; 10–18 min, 50% A in B; 18–25 min, 50% A in B to 100% B; 25–27 min, 100% B; 27–34 min, 100% B–100% C; 34–41 min, 100% C	PDA (450 nm)	[94]
Spinach	Lutein, β-carotene, violaxanthin, neoxanthin	LiChrospher 100 RP-C18 (244 × 4 mm ID, 5 μm) column	(A) MeOH–H_2O (8:2, v/v); (B) EtOAc; 0–2.5 min, 20–22.5% B in A; 2.5–20 min, 22.5–50% B in A; 20–22.5 min, 50% B in A; 22.5–24 min, 50–80% B in A; 24–26 min, 80% B in A; 26–31 min, 80–100% B; 31–34 min, 100% B; flow rate: 1 mL min^{-1}	Vis (440 nm)	[95]
Olive oil	β-Carotene	Tracer Extrasil ODS-2 (150 × 4 mm ID, 5 μm) with a guard column containing the same stationary phase; column temperature: 45°C	(A) MeOH; (B) H_2O; (C) BuOH; 0–3 min, 92:3:5 A/B/C; 3–4 min, 92:8, A/C; 4–9 min, 92:8, A/C	PDA (450 nm)	[96]
Tomato	Lycopene	Ultrasphere (250 × 4.6 mm ID, 5 μm) column	(A) acetonitrile; (B) MeOH; (C) CH_2Cl_2 (43.3:43.3:13.4); flow rate: 1.5 mL min^{-1}	Vis (460 nm)	[78]
Red pepper fruit	β-Carotene	Merck Superspher RP-18 (125 × 4 mm ID, 4 μm) column protected with a LiChrospher 100 RP-18 precolumn (40 × 4 mm ID, 5 μm)	(A) acetonitrile; (B) 2-propanol; (C) EtOAc; 40:40:20 A/B/C, v/v/v; flow rate: 0.8 mL min^{-1}	Vis (450 nm)	[97]

Note: APCI-MS/MS, atmospheric pressure chemical ionization–tandem mass spectrometry; BuOH, 1-butanol; CH_2Cl_2, methylene chloride; ESI-MS/MS, electrospray ionization-tandem mass spectrometry; EtOAc, ethyl acetate; iPrOH, isopropanol; MeOH, methanol; PDA, photodiode-array; *t*-BME, *tert*-butyl-methyl ether; THF, tetrahydrofuran.

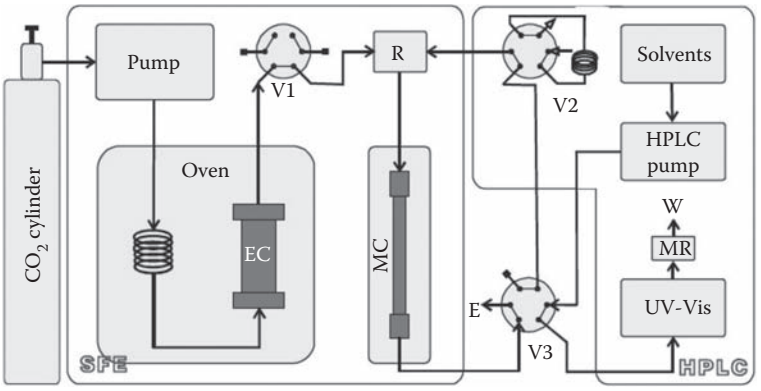


FIGURE 3.6 Supercritical fluid extraction (SFE)–HPLC apparatus displayed in extraction mode. EC, extraction column; R, restrictor; MC, monolithic column placed in the thermostatted chamber; V1, static/dynamic SFE valve; V2, HPLC injection valve with sampling loop; V3, interface valve switching between extraction and analysis mode; E, exhaust; MR, mobile-phase restrictor; W, waste. (From Pól, J., Hyötyläinen, T., Ranta-Aho, O., and Riekkola, M.L., *J. Chromatogr. A*, 1052, 27, 2004. With permission.)

coupled for the analysis of free carotenoids and carotenoid esters in mandarin essential oil. In the first dimension, a Supelcosil LC-SI microbore column, with the mobile phase consisting of *n*-hexane (solvent A) and ethanol (solvent B) used in the linear gradient mode, was employed. In the second dimension, a Chromolith Performance RP-18 monolithic column and two different linear gradients, with 2-propanol as solvent A and water–acetonitrile (2:8, v/v) as solvent B, were utilized. By means of PDA and APCI-MS detection, β-carotene, β-cryptoxanthin, mutatoxanthin, antheraxanthin, and luteoxanthin were identified as the main mandarin carotenoids present in the saponified fraction. This combined technical approach seems to be especially useful to analyze and characterize complex food samples containing carotenoid compounds.

3.5 HPLC ANALYSIS OF PHYTOCHEMICALS IN DIETARY SUPPLEMENTS

3.5.1 HPLC Analysis of Biologically Active Phenolics in Dietary Bee Products: Honey and Propolis

Honey and propolis represent a class of the most famous bee products used in traditional apitherapy but predominantly as nutraceuticals promoting overall health and well-being. They are a rich source of polyphenolic compounds (mainly free and esterified aromatic acids and flavonoids) known as effective antioxidants showing radical-scavenging activities [101,102]. This is also one of the reasons that the majority of honeys and propolis products are reported to possess some important biological activities, such as antibacterial, antifungal, antiviral [103,104], anti-inflammatory [105], and even immunomodulatory and antitumor [106] properties.

The chemical composition of honeys and propolis, their biological properties, and finally their quality depend heavily on the specificity of bioconstituents occurring in the floral components (nectars, pollen, waxes, essential oils, resins) that are collected by honeybees. For example, the bud exudates of poplar trees are the main source of propolis deriving from the moderate climate zone. Therefore, European propolis is known to contain the typical phenolics of poplar buds, such as flavone and flavanone aglycones, phenolic acids, and their esters [107]. Phenolic compounds are also important chemical markers for the floral origin of some honey types, especially in heather, chestnut, eucalyptus, rapeseed, and lime-tree honeys. The role of particular markers, such as hesperetin for citrus honey, kaempferol and its glycosides for rosemary and acacia honey, and quercetin for heather honey, has been confirmed. Abscisic acid, indicated as a marker for heather honey, is also present in significant amounts in rapeseed, lime-tree, and acacia honeys [108,109].

Nowadays, thanks to numerous HPLC analytical methods developed over the last two decades, a detailed chemical analysis of various honey and propolis food supplements is very easy to obtain. Especially the HPLC analysis of the polyphenolic fraction has been suggested to be the most accurate scientific approach for the evaluation of the quality of these dietary supplements. However, because of the complex matrices of honey and propolis products, appropriate sample-preparation procedures for cleanup and isolation of purified phenolic compounds need to be performed. In the latest review on products derived from bees, published by Gómez-Caravaca and coworkers [110], these problems are discussed. In relation to a broad range of honey samples, the procedure of solid-phase extraction (SPE) on Amberlite XAD-2 columns was predominantly used. Aqueous, acidified honey solutions were passed through the columns to retain the phenolic compounds in sorbent beds and to elute them, in the next step, with methanol. Another possibility is the use of SPE cartridges containing C_{18} chemically bonded phases for the isolation of phenolic fractions from ethyl acetate dry honey extracts, dissolved in acidified deionized water. As regards propolis samples, at the beginning of sample preparation, extraction with 70–80% ethanol is especially recommended to obtain dewaxed extracts rich in polyphenolic components. Purified honey or propolis extracts, rich in biologically active polyphenols, are then qualitatively and quantitatively analyzed by HPLC, which is currently the most popular and reliable technique for the characterization of plant phenolic constituents. Table 3.4 presents an overview of the latest HPLC methods (with various mobile and stationary phases as well as detection systems) employed to establish the chromatographic profiles of various classes of honey and propolis polyphenols as markers of their botanical or geographic sample origin. Additionally, it is worth stating that for the last decade the quality control of bee dietary products using HPLC has become much more advanced, comprising important chromatographic studies on the presence of residual, hazardous contaminants in honeys, such as pesticides [111–113] or some antimicrobial drugs (antibiotics [114–118] or sulfonamides [119–121]), used in the treatment of honeybee diseases, as these constituents can cause undesirable or harmful effects in relation to human organisms.

3.5.2 HPLC and Chromatographic Fingerprinting in Quality Control of *Ginkgo biloba* Dietary Supplements

Nowadays, *Ginkgo biloba* leaf extracts are classified as belonging not only among the most popular herbal medicinal products but also among the most commonly used dietary supplements worldwide. Flavonoids and terpene lactones (Figure 3.7) are considered to be the main bioactive components; these are especially helpful in the symptomatic treatment of age-associated memory impairment, dementia (including Alzheimer's disease), and peripheral and cerebral circulation disorders. For this purpose, standardized *G. biloba* extracts are predominantly utilized, containing 24–26% flavonoid glycosides, 5–7% terpene lactones, and less than 5 parts per million allergenic and cytotoxic ginkgolic acids [135].

In the early 1990s, HPLC with UV detection was used to identify the components of *G. biloba* extracts. Hasler and Sticher [136] employed the aforementioned method to analyze 33 flavonoids in this herbal substance. However, the HPLC–UV technique turned out to not be suitable for terpene lactone (ginkgolides, bilobalide) analysis, as these compounds possess poor chromophores, weakly absorbing ultraviolet light at low, nonselective wavelengths (190–220 nm). In the last decade, HPLC coupled with evaporative light scattering detection (ELSD) has enjoyed renewed interest with respect to the determination of terpene lactones and flavonoids in *G. biloba* dietary supplements. ELSD detection provides advantages over UV detection in terms of sensitivity and selectivity and is relatively inexpensive and easy to operate compared to the MS technique. Li and Fitzloff [137] used this method for the simultaneous determination of ginkgolides A, B, C, and J; bilobalide; quercetin; kaempferol; and isorhamnetin in *G. biloba* capsules purchased from a local pharmacy in Chicago. The measurements were performed at 20°C on a Supelco Discovery RP-18 column (250 mm × 4.6 mm ID, 5 μm), protected by a Water Delta-Pak C_{18} guard column, using methanol

TABLE 3.4
HPLC Identification of Phenolic Compounds in Honey and Propolis Samples as Markers of their Botanical and Geographic Origin

Sample Origin	Phenolics	Column	Mobile Phase	Detection	Ref.
Acacia honey from Italy and Slovakia	*Flavonoid floral markers*: kaempferol glycosides	C_{18} LiChrocart (250 × 4 mm ID, 5 μm) protected with a C_{18} LiChrocart (4 × 4 mm) guard column	Gradient elution; (A) H_2O–CH_3COOH (99:1, v/v); (B) MeOH; flow rate: 1 mL min^{-1}	PDA (290 and 360 nm); ESI-MS	[109]
50 samples of Czech honey wines	*Phenolic acids*: vanillic, gallic, protocatechuic, chlorogenic, α- and β-resorcylic, homoprotocatechuic, 2-, 3-, and 4-hydroxyphenyloacetic, *m*-, *o*-, and *p*-coumaric, gentisic, syryngic, ferulic, isoferulic, sinapic; *Other phenolics*: esculetin, vanillin, isovanillin, ethylvanillin, (+) catechin, protocatechuicaldehyde	Gemini C_{18} (150 × 3 mm ID, 3 μm) column kept at 35°C	Gradient elution; (A) 5 mM ammonium acetate in H_2O, pH 3; (B) solvent A–acetonitrile 1:2 (v/v); flow rate: 0.45 mL min^{-1}	EC	[108]
Acacia, Chinese milk vetch, buckwheat, and manuka honeys from Japan	*Phenolic acid esters*: methyl syringate	Discovery RP amide C_{16} (150 × 4.6 mm ID, 5 μm) column kept at 40°C	Gradient and isocratic elution; (A) 0.5% CH_3COOH in H_2O; (B) 0.5% CH_3COOH in MeOH; flow rate: 0.5 mL min^{-1}	EC and ESI-MS	[122]
Strawberry-tree honey from Italy	*Phenolic acid floral marker*: homogentisic acid	Alltima C_{18} (250 × 4.6 mm ID, 5 μm) column with a guard cartridge packed with the same stationary phase	Gradient elution; (A) 5 mM H_2SO_4; (B) MeOH; flow rate: 1.2 mL min^{-1}	UV (291 nm)	[123]
Honeys from Australia	*Main phenolic acids*: gallic, ellagic, chlorogenic, *p*-coumaric (tea tree honey); gallic (crow ash honey); gallic and ellagic (brush box honey); gallic and *p*-coumaric (heath honey); *p*-coumaric and gallic (sunflower honey)	LiChrocart RP-18 (125 × 4 mm ID, 5 μm) column	Gradient elution; (A) H_2O–HCOOH (19:1, v/v); (B) MeOH; flow rate: 1 mL min^{-1}	PDA (290 and 340 nm)	[124]
Eucalyptus honeys from Australia	*Flavonoids*: tricetin, quercetin, quercetin 3-methyl ether, luteolin, myricetin, kaempferol, kaempferol 8-methyl ether, pinocembrin	LiChrocart RP-18 (125 × 4 mm ID, 5 μm) column	Gradient elution; (A) H_2O–HCOOH (19:1, v/v); (B) MeOH; flow rate: 1 mL min^{-1}	PDA (290 and 340 nm)	[125]
Eucalyptus honeys from Australia	*Phenolic acids*: gallic, caffeic, chlorogenic, *p*- and *o*-coumaric, ferulic, ellagic; *Other phenolics*: abscisic acid	LiChrocart RP-18 (125 × 4 mm ID, 5 μm) column	Gradient elution; (A) H_2O–HCOOH (19:1, v/v); (B) MeOH; flow rate: 1 mL min^{-1}	PDA (290 and 340 nm)	[126]

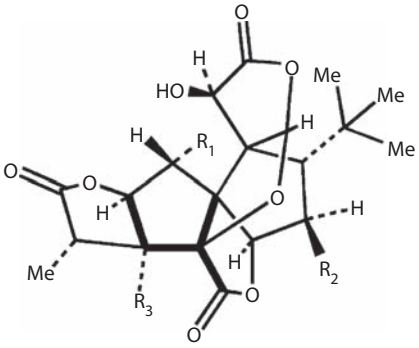
Honeys from Australia and New Zealand	*Main flavonoids*: myricetin, luteolin, tricetin (jelly bush honey); quercetin, isorhamnetin, chrysin, luteolin (manuka honey); *Main phenolic acids*: gallic, *p*- and *o*-coumaric (jelly bush honey); gallic (manuka honey); *Other phenolics*: abscisic acid (jelly bush and manuka honeys)	LiChrocart RP-18 (125 × 4 mm ID, 5 µm) column kept at 35°C	Gradient elution; (A) H_2O–HCOOH (19:1, v/v); (B) MeOH; flow rate: 1 mL min^{-1}	PDA (290 and 340 nm)	[127]
European unifloral honeys	*Flavonoid floral markers*: hesperetin (citrus honey); 8-methoxykaempferol (rosemary honey); quercetin (sunflower honey); *Other phenolic markers*: abscisic acid (acacia honey); *Propolis-derived phenolic constituents*: pinocembrin, pinobanksin, chrysin, caffeic acid, *p*-coumaric acid, ferulic acid	LiChrocart RP-18 (125 × 4 mm ID, 5 µm) column	Gradient elution; (A) H_2O–HCOOH (19:1, v/v); (B) MeOH; flow rate: 1 mL min^{-1}	PDA (290 and 340 nm)	[128]
Eucalyptus honeys from Italy, Portugal, and Spain	*Flavonoid floral markers*: myricetin, tricetin, quercetin, luteolin, kaempferol	LiChrocart RP-18 (125 × 4 mm ID, 5 µm) column	Gradient elution; (A) H_2O–HCOOH (19:1, v/v); (B) MeOH; flow rate: 1 mL min^{-1}	PDA (290 and 340 nm)	[129]
Japanese propolis from Okinawa	*Prenylflavonoid markers*: nymphaeol-A, nymphaeol-B, nymphaeol-C, 3'-geranyl-naringenin, isonymphaeol-B	Capcell Pak UG120 C18 (250 × 4.6 mm ID) column	Gradient elution; (A) 0.1% TFA in H_2O; (B) 0.1% TFA in acetonitrile; flow rate: 1 mL min^{-1}	PDA	[130]
Red Cuban propolis	*Isoflavonoid markers*: pterocarpans, isoflavans, isoflavones	*HPLC-PDA*: µ-Bondapack C-18 (250 × 4.6 mm ID, 10 µm) column *HPLC-HPLC-ESI-MS*: C-18 Hypurity Aquastar (150 × 2 mm ID, 5 µm) column protected by a guard cartridge (4 × 2 mm ID) packed with the same stationary phase	Gradient elution; *HPLC-PDA*: (A) H_2O; (B) MeOH; flow rate: 1 mL min^{-1}; *HPLC-ESI-MS*: (A) H_2O; (B) acetonitrile	PDA (320, 280, and 254 nm); ESI-MS	[131]

(*Continued*)

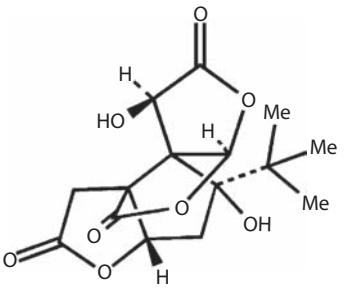
TABLE 3.4 (CONTINUED)
HPLC Identification of Phenolic Compounds in Honey and Propolis Samples as Markers of Their Botanical and Geographic Origin

Sample Origin	Phenolics	Column	Mobile Phase	Detection	Ref.
Propolis of various geographic origin	*Flavonoids*: quercetin, kaempferol, apigenin, pinobanksin, pinobanksin 5-methyl ether, pinobanksin 3-acetate, pinocembrin, chrysin, galangin, tectochrysin; *Phenolic acids*: caffeic, *p*-coumaric, 3,4-dimethoxycinnamic; *Other phenolics*: phenethyl caffeate, cinnamyl caffeate, artepillin C	Capcell Pak ACR 120 C_{18} (250 × 2 mm ID, 5 µm) column	Gradient elution; (A) 0.1% HCOOH in H_2O; (B) 0.08% HCOOH in acetonitrile; flow rate: 1 mL min^{-1}	PDA (195–650 nm); ESI-MS	[102]
Poplar-type propolis from Bulgaria, Italy, and Switzerland	*Flavonoids*: kaempferol, pinocembrin, chrysin, galangin, pinostrobin; *Phenolic acids*: caffeic, *p*-coumaric, ferulic; *Other phenolics*: phenethyl caffeate, isopentyl caffeate, benzyl caffeate	Inertsil 5 ODS-2 (250 × 4.6 mm ID) column with a Chromsphere ODS guard column (10 × 3 mm ID)	Gradient elution; (A) 5% CH_3COOH in H_2O; (B) MeOH; flow rate: 1 mL min^{-1}	UV (290 nm)	[132]
Commercial propolis preparations	*Flavonoids*: quercetin, kaempferol, galangin, naringenin, pinocembrin, chrysin; *Phenolic acids*: caffeic, *o*- and *p*-coumaric	Symmetry C_{18} (220 × 4.6 mm ID, 5 µm) column	Gradient elution; (A) 30 mM NaH_2PO_4, pH 3; (B) acetonitrile; flow rate: 1.5 mL min^{-1}	PDA (265, 290, and 360 nm)	[133]
Brazilian propolis	*Flavonoids*: kaempferol, pinobanksin, pinobanksin 3-acetate, apigenin, isosakuranetin, pinocembrin, chrysin, galangin, tectochrysin; *Phenolic acids*: coumaric, ferulic, dimethylallyl caffeic acid	YMC Pack ODS-A RP-18 (250 × 4.6 mm ID, 5 µm) column	Gradient elution; (A) H_2O; (B) MeOH; flow rate: 1 mL min^{-1}	PDA (268 nm)	[134]

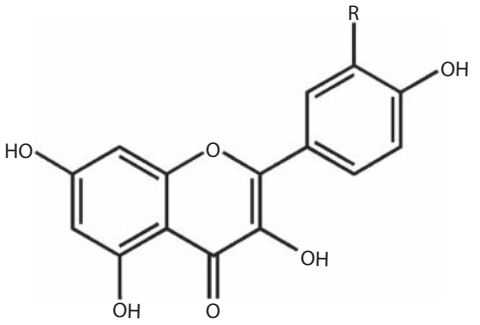
Note: EC, electrochemical; ESI-MS, electrospray ionization–mass spectrometry; MeOH, methanol; PDA, photodiode-array; TFA, trifluoroacetic acid.



Ginkgolide A: $R_1 = R_2 = H$, $R_3 = OH$
Ginkgolide B: $R_1 = R_3 = OH$, $R_2 = H$
Ginkgolide C: $R_1 = R_2 = R_3 = OH$
Ginkgolide J: $R_1 = H$, $R_2 = R_3 = OH$



Bilobalide



Kaempferol: R = H
Quercetin: R = OH:
Isorhamnetin: R = OMe:

FIGURE 3.7 Structure of ginkgolides A, B, C, and J; bilobalide; quercetin; kaempferol; quercetin; and isorhamnetin. (From Li, W. and Fitzloff, J.F., *J. Pharm. Biomed. Anal.*, 30, 70, 2002. With permission.)

(containing 0.05% TFA) and water (containing 5% methanol and 0.05% TFA) as the mobile phase over 35 min. Nebulization of the chromatographic eluent was provided by a stream of pressured air at 2.9 bar, and the evaporating temperature was set at 61°C (Figure 3.8).

Herring [138] used the same method for rapid (14 min) determination of terpene lactones in capsule, tablet, and liquid forms of *G. biloba* as a standardized dietary supplement. An Alltima C_{18} column (100 mm × 4.6 mm ID, 3 μm) and a gradient mobile-phase system consisting of 0.1% TFA in water (solvent A) and 0.1% TFA in methanol (solvent A) have been used. Lang and coworkers [139] developed sample-preparation procedures for determination of *G. biloba* terpene lactones, including bilobalide and ginkgolides A, B, C, and J, in various sample matrices, comprising beverages, snacks, and capsules containing *G. biloba*. After preliminary ultrasound-assisted extraction of *G. biloba* leaves and capsules with boiling water (tea bags) or 5% KH_2PO_4 aqueous solution, samples were filtered, and NaCl was added to approximately 30% (w/v). Finally, terpene lactones contained in aqueous extracts were subjected to solvent extraction with ethyl acetate and tetrahydrofuran. After evaporation of the organic phase, the aforementioned compounds were dissolved in methanol and separated on a Phenomenax C_{18} (250 × 4.6 mm ID) column using a gradient of water and a mixture of methanol and isobutanol together with ELSD. Dubber and Kanfer [140] recently published a precise, rapid, and fully validated (according to the U.S. Pharmacopeia standards) RP-HPLC-ELSD method for the quantitative determination of the terpene trilactones: ginkgolides A, B, C, and J and the sesquiterpene bilobalide in *G. biloba* solid oral-dosage forms. Compound separation was carried

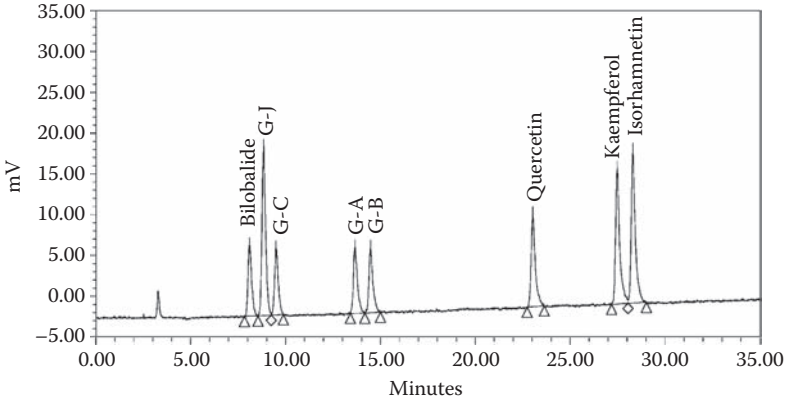


FIGURE 3.8 A typical HPLC–evaporate light scattering detector (ELSD) chromatogram of a standard mixture with 200 ng of ginkgolides A, B, and C (labeled G-A, G-B, and G-C); bilobalide; quercetin; kaempferol; and isorhamnetin and 350 ng of ginkgolide J (labeled G-J) on column. (From Li, W. and Fitzloff, J.F., *J. Pharm. Biomed. Anal*., 30, 72, 2002. With permission.)

out on a minibore Phenomenax Luna C_{18} (250 × 2 mm, 5 μm) column, maintained at a temperature of 45°C, using a simple gradient of methanol and water within 14 min.

Taking into account other detection techniques, MS has proved to be another valuable method due to its sensitivity, whereas MS/MS facilitated exclusive analysis of selected peaks of interest in *G. biloba* extracts. Ding et al. [141] optimized simultaneous determination of 10 major components—bilobalide; ginkgolides A, B, and C; quercetin; kaempferol; isorhamnetin; rutin hydrate; quercetin-3-β-D-glucoside; and quercitrin hydrate (Figure 3.9)—in the *G. biloba* nutritional supplement using a Luna C_{18} RP column, a mobile phase composed of 0.1% (v/v) acetic acid in water (solvent A) and a 1:1 (v/v) mixture of acetonitrile and methanol (solvent B) and ESI-MS in the negative ion mode. De Jager and coworkers [142] developed a method comprising HPLC separation of ginkgolides A, B, C, and J and bilobalide in five *G. biloba* dietary supplements using a Hyperclone ODS C_{18} column, a mobile-phase solvent gradient consisting of methanol and water, and APCI-MS in the negative ionization mode. In 2005, two papers were published describing the use of RP-HPLC coupled with MS/MS for analysis of selected flavonol glycosides (rutin, quercitrin) and aglycones (quercetin, kaempferol, isorhamnetin) [143] as well as terpenoid lactones [144] in *G. biloba* oral dietary supplements.

Because of the previously described structural and quantitative complexity of *G. biloba*'s active constituents, the common analytical problem, which also applies to dietary products derived from this herbal substance, is the lack of reference standards for some pharmacologically active components. For this reason, the quality control of *G. biloba* extracts has often been realized using chromatographic fingerprinting (accepted by the WHO) with the employment of various chromatographic (HPLC, gas chromatography [GC], thin-layer chromatography [TLC]) and spectral (MS and NMR) techniques, in which the experimental data from the chemical analysis of different preparations might be compared without accurate quantification or identification of the individual compounds. Chen and coworkers [145] used HPLC-UV fingerprinting for the examination of chromatographic profiles of flavonoids present in methanol–water extracts from 14 commercial *G. biloba* dietary supplements, 1 American Herbal Pharmacopoeia–verified *G. biloba* leaf sample, and 3 *G. biloba* standard reference materials from the National Institute of Standards and Technology. To summarize the multivariate data obtained, principal-component analysis (PCA) was used, which revealed significant variations in the commercial products as regards the relative content of individual flavonols. Ding and coworkers [146] employed a much more advanced HPLC-ESI-MS/MS method to identify simultaneously more than 70 flavonoid and terpene lactone components in five commercial *G. biloba* dietary supplements. In the summary of the work, the authors stated that the method could be regarded as complementary to specific quantitative analysis of some bioactive constituents in the quality control of *G. biloba* products.

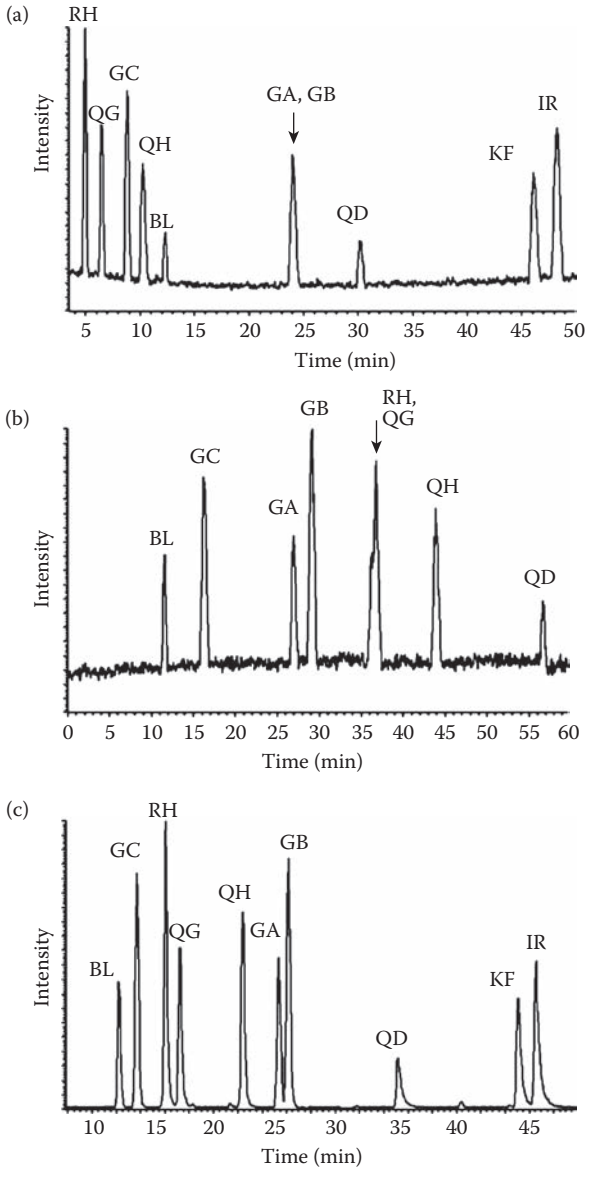


FIGURE 3.9 Comparison of liquid chromatography–mass spectrometry (LC-MS) chromatograms of a mixture of 10 standard *Ginkgo* components: ginkgolide A (GA), ginkgolide B (GB), ginkgolide C (GC), bilobalide (BL), quercetin dehydrate (QD), quercetin-3-β-D-glucoside (QG), quercitrin hydrate (QH), kaempferol (KF), isorhamnetin (IR), and rutin hydrate (RH). Mobile phases tested: (a) 0.1% acetic acid (solvent A), acetonitrile (solvent B), 20% B for 5 min, 20–30% B for 40 min, 30–35% B for 10 min; (b) 0.1% acetic acid (solvent A), methanol (solvent B), 25–50% B in 60 min; (c) 0.1% acetic acid (solvent A), 1:1 (v/v) mixture of acetonitrile and methanol (solvent B), gradient from 20% to 45% B over 50 min, then 100% B for 5 min and equilibration for 10 min. (From Ding, S., Dudley, E., Plummer, S., Tang, J., Newton, R.P., and Brenton, A.G., *Rapid Commun. Mass Spectrom.*, 20, 2757, 2006. With permission.)

3.5.3 HPLC in Qualitation and Quantitation of Phytoestrogenic Isoflavones

Natural compounds possessing estrogenic activity, called phytoestrogens, are especially abundant in the Leguminosae family. Nowadays these phytochemicals are essential for preventing menopausal symptoms and some related diseases such as osteoporosis, atherosclerosis, and even certain

sex-hormone-related (breast, ovary, prostate) cancers [147,148]. In this group, soybean (*Glycine max* (L.) Merrill), kudzu (*Pueraria lobata* L.), and clover (*Trifolium* L.) isoflavones (genistein, daidzein, formononetin, biochanin A, and glycytein and their glycosyl, acetyl, or malonyl forms as well as puerarin derivatives that are characteristic for kudzu), as the constituents of foods and numerous dietary supplements, have attracted scientific attention, and a great number of studies have been undertaken for their chromatographic separation and quantitation [149,150].

Based on the data published by Rostagno and coworkers [151], various extraction procedures have been utilized for the micropreparative isolation of isoflavones from plant material or dietary supplements. Typically, these compounds have been extracted using aqueous alcohols (methanol, ethanol), acetone, and acetonitrile, with or without the addition of hydrochloric acid as a hydrolytic agent, by simple mix-stirring at room temperature. To enhance extraction efficiency, ultrasound-assisted extraction (UAE) or microwave-assisted extraction (MAE) have also been employed. With regard to separation chromatographic techniques, HPLC on reversed-phase columns has been used, with gradient eluent systems consisting of methanol or acetonitrile and acidified (with formic, acetic, trifluoroacetic, or phosphoric acids) water, as well as UV and/or fluorescence detection [152]. Isoflavones, possessing numerous chromophores, readily absorb UV light with a maximum range from 250 to 270 nm, while fluorescence detection provides high sensitivity and specificity in the HPLC separation of these compounds. Additionally, isoflavones, as electroactive phenolics, may be effectively analyzed by means of electrochemical detection. Recently, real progress in the quality control of dietary supplements containing isoflavone phytoestrogens has been realized by coupling MS with HPLC. ESI and APCI interfaces are predominantly used. The application of the triple quadrupole detectors enables the obtaining of MS/MS spectra and performing of multiple reaction ion monitoring (MRM), which is very useful in the precise and selective quantitation of isoflavones [153]. Current data on the application of the HPLC method, coupled with various detection techniques, to the qualitative and quantitative analysis of isoflavone phytoestrogens in dietary supplements and food samples have been collected in Table 3.5.

3.5.4 Quality Control of Dietary Supplements Containing Soybean Lecithins by HPLC

Apart from biologically active isoflavones, described in the previous section, soybeans also contain a phospholipid complex (in crude oil fraction) commonly called lecithin. Soy-derived lecithin, as a functional ingredient of foods and numerous dietary supplements, significantly lowers LDL cholesterol and triglyceride levels and increases the concentration of antiatherogenic HDL cholesterol fractions in blood [168]. Phospholipids are also the principal components of the myelin neuron sheaths and hepatocytes; thus they may be regarded as neuro- and hepatoprotective plant agents. Commercial soy lecithins usually constitute the mixture of five main phospholipids: phosphatidylcholine (PC), phosphatidylethanoloamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA). Generally, each phospholipid contains a glycerol molecule,two hydroxyl groups of which are bonded to two fatty acids, while the third one is linked to the phosphate, which may be further esterified with other organic molecules, for example, choline, ethanoloamine, inositol, or serine.

As fatlike compounds possessing bipolar character, phospholipids should be extracted from plant material by means of both polar and nonpolar organic solvents. Traditionally, mixtures of chloroform and methanol have been recommended. Crude extracts have been further purified from nonlipid components on Sephadex G-25 columns [169]. Because of the lipophilic character of phospholipids, Snyder et al. [170] regarded SFE as a rapid and efficient technique for isolating these compounds from food samples. Another resolution of this problem was the employment of preparative HPLC for the fractionation of soybean phospholipids. Kang and Row [171] optimized mobile-phase composition for normal-phase (NP) preparative HPLC separation of PE, PI, and PC from soybeans on a large-scale. Hexane, methanol, and isopropanol were used as mobile-phase components in linear gradient mode. The stationary phases consisted of four different sizes of preparative silica packings: 5–20 μm, 15 μm, 25–40 μm, and 40–63 μm. The 15 μm particle size gave the best separation of the soy lecithin phospholipids examined.

TABLE 3.5
HPLC Analysis of Phytoestrogenic Isoflavones in Dietary Supplements and Foods

Sample	Mobile Phase	Column	Detection	Ref.
Soy-based infant formulas	Gradient elution; (A) 0.1% CH_3COOH in H_2O; (B) 0.1% CH_3COOH in acetonitrile; flow rate: 1 mL min^{-1} for 5 min and 1.5 mL min^{-1} for the next 40 min	YMC-Pack ODS-AM-303 (250 × 4.6 mm ID, 5 µm) column maintained at 34°C	PDA (from 200 to 350 nm)	[154]
Soy-based dietary supplements	Gradient elution; (A) 50 mM sodium acetate buffer, pH 5–MeOH, 80:20 v/v; (B) 50 mM sodium acetate buffer, pH 5–MeOH–acetonitrile, 40:40:20 v/v/v; flow rate: 0.3 mL min^{-1}	Inertsil ODS-3 (150 × 3 mm ID, 5 µm) with a Quick Release C_{18} (10 × 3 mm ID, 5 µm) guard column kept at 37°C	EC	[155]
Kudzu dietary supplements	Gradient elution; *HPLC-PDA*: (A) 10% aqueous acetonitrile–0.1% TFA; (B) 90% aqueous acetonitrile–0.1% TFA; flow rate: 1.5 mL min^{-1}; *HPLC-MS/MS*: (A) 10 mM NH_4OAc in H_2O; (B) 10 mM NH_4OAc in acetonitrile; flow rate: 0.1 mL min^{-1}	*HPLC-PDA*: Aquapore C_8 (220 × 4.6 mm ID, 300 Å pore size) column with a RP-8 New Guard (15 × 3.2 mm ID) column maintained at 24°C; *HPLC-MS/MS*: Aquapore C_8 (150 × 2.1 mm ID) column	PDA (262 nm); ESI-MS/MS	[156]
Soy, red clover, and kudzu dietary supplements	Gradient elution; (A) 0.1% CH_3COOH in H_2O; (B) 0.1% CH_3COOH in acetonitrile; flow rate: 1 mL min^{-1}	Nova-Pak C_{18} (150 × 3.9 mm ID, 4 µm) column kept at 40°C	PDA (260 nm)	[157]
Soy extracts	Gradient elution; (A) 0.1% CH_3COOH in H_2O; (B) acetonitrile; flow rate: changeable during analysis from 3 to 4 mL min^{-1}	Two linked Chromolith Performance RP-18e (100 × 4.6 mm) columns	PDA (from 200 to 400 nm); ESI-MS	[158]
Tofu, soybeans, and isoflavone supplements	Preparative HPLC with gradient elution; (A) 0.1% CH_3COOH in H_2O; (B) acetonitrile; flow rate: 5 mL min^{-1}	Supelcosil SPLC-18-DB (250 × 10 mm ID, 5 µm) preparative column	UV (254 nm)	[159]
Soybean products	Gradient elution; (A) 50 mM sodium acetate buffer, pH 5–MeOH, 80:20 v/v; (B) 50 mM sodium acetate buffer, pH 5–MeOH–acetonitrile, 40:40:20 v/v/v; flow rate: 0.3 mL min^{-1}	Inertsil ODS-3 (150 × 3 mm ID, 5 µm) protected with a Quick Release C_{18} (10 × 3 mm ID, 5 µm) guard column kept at 37°C	EC	[160]
Soybean seeds	Gradient elution; (A) acetonitrile; (B) CH_3COOH–H_2O, 10:90 v/v; flow rate: 0.8 mL min^{-1}	Nova-Pak C_{18} (150 × 3.9 mm ID, 4 µm) with an Adsorbosphere C_{18} (10 × 4.6 mm ID, 5 µm) guard column	PDA (260 and 342 nm) FL (Ex = 340 nm; Em = 418 nm)	[161]

(*Continued*)

TABLE 3.5 (CONTINUED)
HPLC Analysis of Phytoestrogenic Isoflavones in Dietary Supplements and Foods

Sample	Mobile Phase	Column	Detection	Ref.
Soybean seeds (edamame and tofu varieties)	Gradient elution; (A) 0.1% HCOOH in H_2O; (B) 0.1% HCOOH in acetonitrile; flow rate: 0.8 mL min^{-1}	Phenomenax ODS (150 × 3.2 mm ID, 5 μm) column maintained at 25°C	PDA (254 nm); ESI-MS/MS	[162]
Korean soybean seeds	Gradient elution; (A) 0.1% CH_3COOH in H_2O; (B) 0.1% CH_3COOH in acetonitrile; flow rate: 1 mL min^{-1}	YMC-Pack ODS-AM-303 (250 × 4.6 mm ID, 5 μm) column	PDA (254 nm)	[163]
Soybean seeds from India and Bulgaria	Gradient elution; (A) 0.1% CH_3COOH in H_2O; (B) 0.1% CH_3COOH in acetonitrile; flow rate: 1 mL min^{-1}	Bondapak C_{18} (250 × 4.6 mm ID, 5 μm) column	UV (256 nm)	[164]
Soy bread	Gradient elution; (A) 1% CH_3COOH in H_2O; (B) acetonitrile; flow rate: 0.6 mL min^{-1}	Nova-Pak C_{18} (150 × 3.9 mm ID, 4 μm) column	PDA (260 nm)	[165]
Tofu	Gradient elution; (A) 0.1% CH_3COOH in H_2O; (B) 0.1% CH_3COOH in acetonitrile; flow rate: 1 mL min^{-1}	Phenomenax Luna C_{18} (250 × 4 mm ID, 5 μm) column	UV (254 nm)	[166]
Soybean sprouts	Gradient elution; (A) 0.1% CH_3COOH in H_2O; (B) 0.1% CH_3COOH in acetonitrile; flow rate: 1 mL min^{-1}	YMC-Pack ODS-AM-303 (250 × 4.6 mm ID, 5 μm) column	UV (254 nm)	[167]

Note: EC, electrochemical; Ex, excitation wavelength; Em, emission wavelength; ESI-MS/MS, electrospray ionization–tandem mass spectrometry; FL, fluorescence; MeOH, methanol; PDA, photodiode-array; TFA, trifluoroacetic acid.

Hanras and Perrin [172] carried out gram-scale preparative HPLC of PC, PE, PA, and PI from soybean lecithins. Successive steps of this procedure comprised the preparation of deoiled soybean lecithin using acetone, and its further alcohol fractionation, followed by separation of particular phospholipids on silica columns with increasing internal diameters and elution performed with the organic mobile phase without acidic or basic components. With the largest 50 mm ID column, 2.1 g of phospholipids (of high purity, >99%) were separated. In 1992, the International Union of Pure and Applied Chemistry (IUPAC) published the results of a collaborative study and presented a standardized method for determining the phospholipid profile of soy lecithins by HPLC [173]. The samples studied were deoiled commercial soybean lecithin concentrates that had been analyzed in 12 laboratories representing nine countries. For the separation of phospholipids (PE, PA, PI, and PC), a stainless steel column (200 × 4.6 mm ID) packed with spherical silica, a mobile phase prepared by mixing *n*-hexane, 2-propanol, and acetate buffer in the proportion 8:8:1 (v/v/v) and used at a flow rate of 2 mL min^{-1}, and UV detection (at 206 nm) have been employed. In 1996, Szücs and coworkers [174] also applied the aforementioned IUPAC method to the analysis of soy lecithin phospholipids. The only exception was that a shorter column (100 × 4 mm ID) than advised by IUPAC was used, and the flow rate was adjusted to 1 mL min^{-1}. However, as the authors of the paper stated, the IUPAC method completely ignored the presence of PS, being the minor phospholipid component of soybean lecithins.

Van der Meeren and coworkers [175] announced in their study a similar separation problem. They used both semipreparative and analytical NP-HPLC with a stepwise gradient of hexane, 2-propanol, and water for the analysis of soybean phospholipids. The obtained results indicated that only partial resolution of these compounds is possible because of peak deformation caused by the influence of structurally differentiated fatty acid components present in phospholipid molecules, which were retained more weakly or strongly during normal-phase chromatography. Another problem was the lack of chromophores, resulting in the nonspecific absorption of phospholipids in the range of 200–210 nm, which complicated UV detection of these compounds.

Therefore, recently, HPLC with ELSD has been documented as an effective analytical method giving better reproducibility, higher sensitivity, and lower interferences of baseline drift during gradient elution in comparison with UV detectors. Zhang et al. [176] developed the NP-HPLC-ELSD method for quantification of soybean phospholipids in degummed oil residue using 5 μm silica gel as the stationary phase and a ternary gradient with *n*-hexane, isopropanol, and water as the mobile phase. Neutral lipids and glycolipids together with commonly known (PE, PI, PC) and rarer soybean phospholipids (sphingomyelin, lysophosphatidylcholine) were well resolved (Figure 3.10). Additionally, under the same chromatographic conditions, the comparison was made between ELSD, UV, and refractive index (RI) detectors, which confirmed that ELSD was the most useful in the HPLC qualitation and quantitation of this group of natural compounds. Taking into account a group of more sophisticated analytical techniques, Careri and coworkers [177] developed the NP-LC-MS technique using an ionspray (ISP) interface to identify phospholipids from soybean products. PC and PE were detected in the form of positive ions generated by ISP, whereas acidic phospholipid compounds (PI) were analyzed using the negative-ion ISP. For more detailed quality control and better characterization of the fatty acid chains and the polar head group of soybean phospholipids, tandem mass spectrometry (ISP-MS/MS) was also employed, giving the possibility of monitoring specific ions of the analytes and enhancing both method selectivity and sensitivity.

3.5.5 Application of HPLC to the Analysis of Pharmacologically Active Lignans in Food Supplements

Numerous dietary supplements contain pharmacologically active lignans, representing the class of phenylpropane derivatives and occurring in flaxseed (*Linum usitatissimum* L.) and *Schisandra chinensis* fruit. Flaxseed lignans, mainly secoisolariciresinol and matairesinol, possess phytoestrogenic

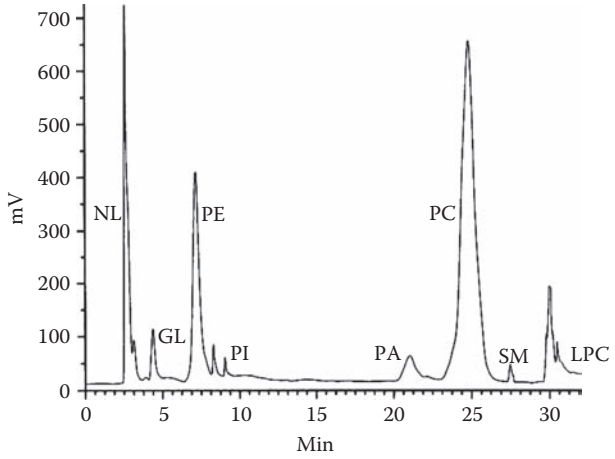


FIGURE 3.10 Chromatogram of soybean lecithin sample with evaporative light scattering detection (ELSD). Peak identification: NL, neutral lipids; GL, glycolipids; PE, phosphatidylethanoloamine; PI, phosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; SM, sphingomyelin; LPC, lysophosphatidylcholine. (From Zhang, Y., Yang, Y., and Ren, Q., *J. Liq. Chromatogr. Related Technol*., 28, 1339, 2005. With permission.)

properties. However, they are not estrogenic by themselves, but they are converted by gut microflora to the mammalian lignans, enterodiol and enterolactone, respectively, which reveal estrogenic activity [147]. Therefore, flaxseed dietary supplements may be used to alleviate some menopausal symptoms, such as hypercholesterolemia or osteoporosis [178,179]. The lignan constituents of *S. chinensis* fruits are dibenzo[*a,c*]cyclooctadiene derivatives (schisandrin, schisantherines, gomisins), which are especially famous for adaptogenic and antihepatotoxic properties [180]. Because of the differences in the chemical structure of flaxseed and *S. chisensis* lignans, the method of their isolation and especially chromatographic analysis varies to some extent. However, standard extraction procedures for lignans are similar and comprise extraction in a Soxhlet apparatus or percolation from plant material using methanol, ethanol, or acetone. Sometimes, it is advisable to do preliminary sequential extraction to remove lipohilic ballast compounds with nonpolar solvents such as hexane, petroleum ether, or dichlorometane. Special pretreatment is required for some flaxseed lignan glycosides present in plant material as ester-linked oligomeres or polymers, which should be subjected to alkaline or acid hydrolysis to release free aglycones and simplify further chromatographic analysis of extracts [181,182]. The important method of choice for extraction of more hydrophobic dibenzo[*a,c*]cyclooctadiene lignans of *S. chisensis* is SFE, which under optimized conditions offers cleaner extracts and utilizes no or only minimal amounts of organic solvents as polar modifiers [180].

For chromatographic analysis of lignans, which are generally medium-polar compounds, RP-HPLC combined with various detection techniques is probably the most frequently used analytical technique. As regards the isolation and characterization of phytoestrogenic lignans (isolariciresinol, pinoresinol, secoisolariciresinol, and mataresinol) from defatted flaxseed meal, HPLC on a semipreparative C_{18} column with a water–acetic acid–acetonitrile gradient eluent system and PDA detection (at 280 nm) has been used [183]. In another study, a combination of classical RP- and chiral HPLC for the quality control and analysis of flaxeed lignans led to the separation of secoisolariciresinol enantiomers and documented that one of the isomeric forms was always predominant in this herbal substance [184]. Further studies on the chemodiversity of lignans in the genus *Linum* revealed the necessity to develop HPLC methods with more advanced detection systems for fast identification and structural characterization of these compounds. German scientists established chemical structures of 24 flaxseed lignans of different skeletal types using a combined HPLC-UV/PDA and HPLC-ESI-MS technique [185]. In a review paper on lignans of the genus

Schisandra, the authors described various modes of HPLC analysis used in the quality control of *Schisandra* fruit, which included applications where RP-HPLC methods with a methanol–water eluent system, or a mobile phase containing acetonitrile, or methanol and acetonitrile had been developed [180].

More recent papers have confirmed the suitability of these systems in the separation of *Schisandra* lignans. For example, a newly improved RP-HPLC validated method was employed for quantification of eight lignans in *S. chinensis* fruit and 13 dietary supplements containing this herbal substance. Compounds were separated with acetonitrile–water–methanol gradient on a Phenomenax Luna C_{18} reversed-phase column with detection at 215 nm [186]. Other authors used microwave-assisted extraction for isolation of lignan constituents from 10 samples of *Schisandra* fruit collected from different regions in China. HPLC fingerprint studies have been further performed using the optimum gradient of acetonitrile and water as a mobile phase and a Diamonsil C_{18} column as a stationary phase, as well as a broad range (210–400 nm) of spectra that have been collected on a PDA detector. The method turned out to be suitable for the quick analysis of sample differentiation depending on its geographic origin as well as for its quality control [187]. Scientists from Taiwan elaborated a novel, high-throughput HPLC screening method for dibenzo[*a,c*]cyclooctadiene lignans in ultrasonic and supercritical fluid extracts from *S. chinensis* fruits [188]. A Phenomenax Luna C_{18} column and a gradient mobile-phase system consisting of water and acetonitrile were employed for the chromatographic analyses. Both UV (at 225 nm) and ESI–ion trap–MS/MS were used as detection techniques (Figure 3.11). The application of advanced spectral methods enabled the introduction of effective HPLC fingerprinting for quality control of *Schisandra* fruits and eliminated any false positive identification of lignans in the samples examined.

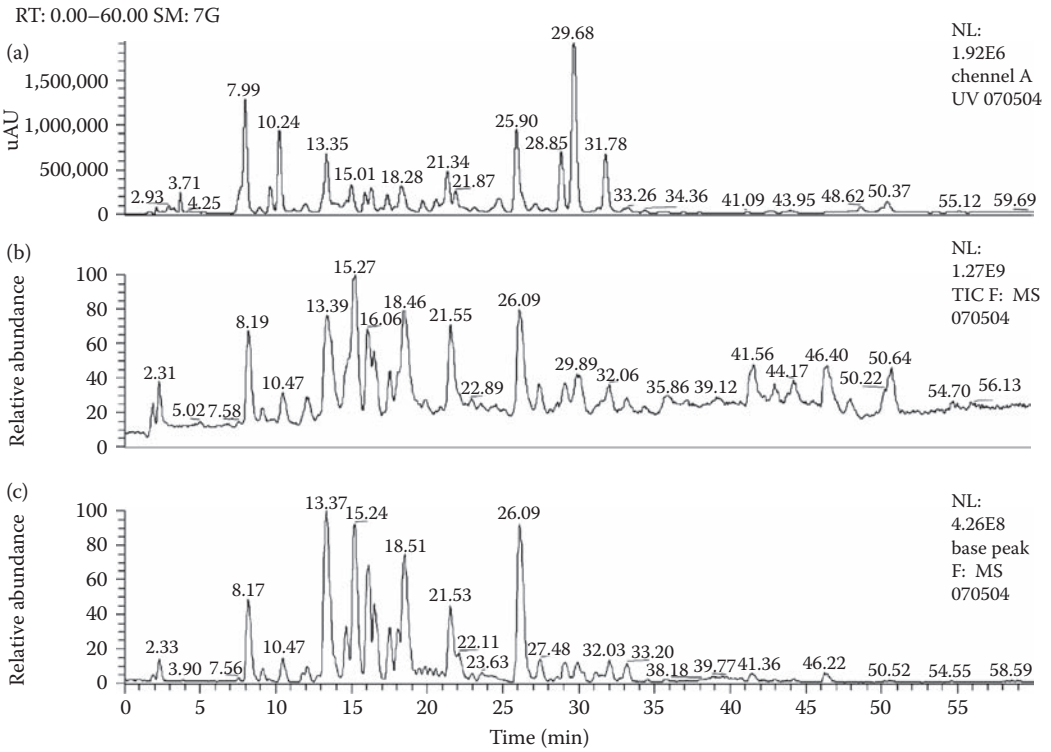


FIGURE 3.11 HPLC-UV and HPLC–electrospray ionization–mass spectrometry (ESI-MS) chromatograms of a modified-ultrasonic extract from *Schisandra chinensis* Baill. (a) Detection, UV = 225 nm; (b) positive ESI-MS total ion chromatogram; (c) positive ESI-MS basic peak chromatogram. (From Wang, M.C., Lai, Y.C., and Chang, C.L., *J. Sep. Sci*., 31, 1328, 2008. With permission.)

3.6 CONCLUSION

In the last decade, the quality control and monitoring of hazardous residues in foods and dietary supplements containing botanical-sourced constituents have become major topics of interest for international and local agencies dealing with the problem of human nutrition and health. The effects of growing conditions, processing, storage, genetics, and other factors concerning the composition and the concentration of plant active components are important issues to be discussed. In the field of quality control, there is a strong demand for new analytical techniques capable of providing sufficient information about various classes of phytochemicals present in foods and dietary supplements. Consequently, faster, more convenient, powerful, and cheaper analytical procedures are required by chemists and quality-control laboratories to meet these demands. In this chapter, important aspects of the application of HPLC method and related spectral techniques to the analysis and qualitative evaluation of some food products and dietary supplements have been discussed.

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4 HPLC in Chemosystematics

Renate Spitaler and Christian Zidorn

CONTENTS

4.1 INTRODUCTION

Chemosystematics is an interdisciplinary field of natural sciences investigating the distribution of natural products within all major groups of living organisms (archaea, bacteria, fungi, plants, and animals). Many primary metabolites are shared by most forms of life (with the notable exception of many archaea), and studies in chemosystematics aiming at the elucidation of relationships between taxa therefore usually focus on secondary metabolites [1]. *Chemosystematics* (adjective: *chemosystematic*) is the name for this discipline preferred here following the arguments put forward by Merxmüller in his critical discussion of the subject [2]. A recent ISI Web of Knowledge search (accessed on September 3, 2008) revealed that the terms *chemotaxonomic/chemotaxonomy*, with 3758 hits, were much more widely used than the term *chemosystematic*(*s*), with only 675 hits. This might partially be explained by the fact that the most eminent figure in the field, Robert Hegnauer, also favored the term *chemotaxonomy* [3]. Interestingly, 4363 papers were found when combining the hits for either of the two search terms, and only 70 papers were found when both search terms *chemosystematic*(*s*) and *chemotaxonomy*(*ic*) were combined with AND.

When searching the literature for papers dealing with comparative investigations on the distribution of secondary metabolites, the terms *biochemical systematics*, *chemical systematics*, *chemical taxonomy*, *chemotaxonomic*, *chemotaxonomy*, *comparative natural products chemistry*, and

comparative phytochemistry all have to be considered in the search strategy. The proportion of false hits—that is, papers dealing with different subjects—are high, especially when using binary search terms. The terms *biochemical systematics* and *biochemical taxonomy* usually refer—today—to molecular techniques like sequencing of DNA fragments.

Chemosystematics was initially mostly performed with comparative analyses using paper chromatography (PC) or thin-layer chromatography (TLC) [4,5]. These techniques do not require expensive instrumentation and were thus widely available to botanists and other scientists working in the emerging field of chemosystematics [6]. Since its development in the 1960s, high performance liquid chromatography (HPLC), initially also termed high pressure liquid chromatography, has become the most important instrumental separation technique for nonvolatile secondary metabolites [7]. Soon after its development HPLC was also applied to the separation of natural products [8–10]. Today, HPLC is still the most important technique in the analysis of natural products, except for the investigation of essential oils, which is dominated by gas chromatography (GC) [11]. Additionally, TLC is still used extensively in areas where fast and cheap results are more important than quantitative and reproducible results, that is, for in-process-control in industry and in natural-product isolation in academic settings [12,13]. The importance of HPLC compared to other analytical techniques is depicted in Figure 4.1. Note, however, that the displayed result is partially misleading as early chemosystematic/chemotaxonomic studies were always based on either PC or TLC, and this fact is often not stated in either the abstract or the keywords of the respective papers.

The first explicit application of HPLC in plant chemosystematics was published in 1984 [14]. Chemosystematics has lost some of its appeal to taxonomists with the advent of DNA-based phylogenetic methods. However, the number of papers dealing with the topic of chemosystematics in the broadest sense (Figure 4.2) and with chemosystematics based on HPLC (Figure 4.3) are still growing. Interestingly, only two journals, *Biochemical Systematics and Ecology* with 44 and *Phytochemistry* with 40 papers, contribute more than 10% of the 262 papers (between 1984 and September 2008) dealing explicitly with chemosystematics/chemotaxonomy and HPLC. The other papers are scattered among 93 other journals.

The history of chemosystematics in general, its application to problems of systematic botany, and the coding and interpretation of data have been described in some detail before [1]. The 10 countries contributing the most papers dealing with HPLC and chemosystematics up to now are displayed in Figure 4.4. It is obvious that Germany, the United States, and the United Kingdom dominated the field in the last 25 years, and not the countries with the highest biodiversity and chemodiversity. However, this is likely to change as HPLC-based analytical systems are becoming more widely available in those parts of the world where biological and chemical diversity is highest.

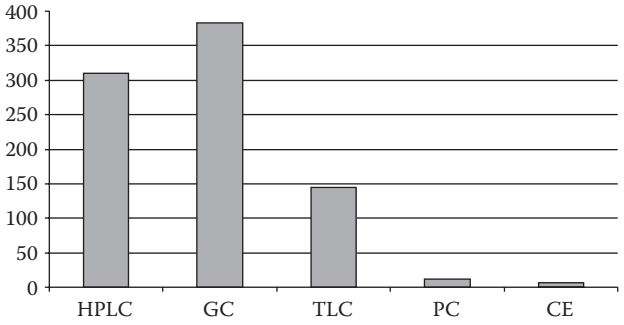

FIGURE 4.1 Hits for the topic "chemosystematics" in the broadest sense (i.e., including the terms *chemosystematic*, *chemotaxonomy*, and *chemotaxonomic*) combined with the most important analytical techniques in the ISI Web of Knowledge database (accessed September 3, 2008). Abbreviations: HPLC, high performance liquid chromatography; GC, gas chromatograph; TLC, thin-layer chromatography; PC, paper chromatography; CE, capillary electrophoresis.

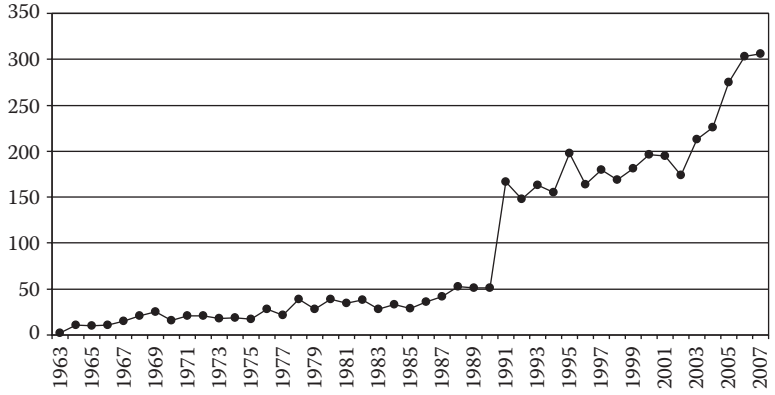

FIGURE 4.2 The number of articles dealing with the topics chemosystematic/chemosystematics and chemotaxonomic/chemotaxonomy from 1963 (first record) to 2007 based on the ISI Web of Knowledge database (accessed September 3, 2008).

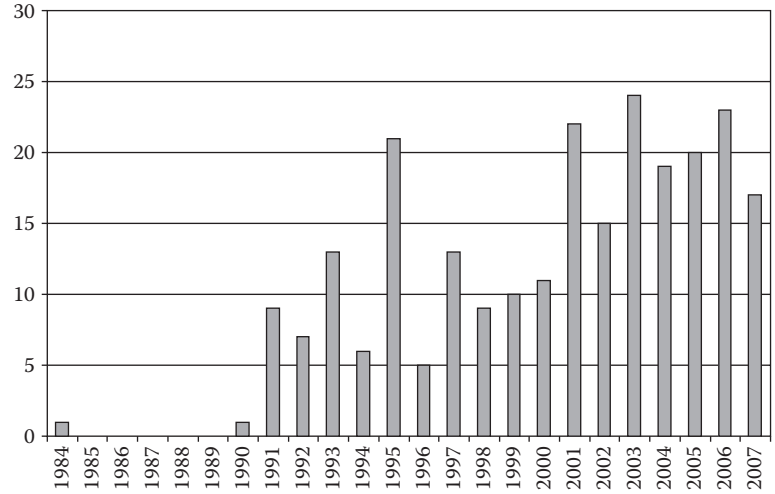

FIGURE 4.3 The number of articles dealing with chemosystematics in the broadest sense (see terms listed in Figure 4.2) and HPLC from 1984 (first record) to 2007 based on the ISI Web of Knowledge database (accessed September 3, 2008).

Thus, countries like Brazil and China, now ranking in positions 10 and 9, respectively, are likely to be the most important contributors to the field in the future.

4.2 HPLC IN NATURAL-PRODUCT ANALYSIS

Currently, HPLC is the most versatile and most widely applied technique in the analysis of natural products. HPLC's main advantage is that basically all compounds soluble in water or one of the many organic solvents employed for HPLC can be analyzed. In most cases compounds are detected with ultraviolet (UV) detectors or photodiode array detectors (DAD). Recently, the availability of a new generation of evaporative light scattering detectors (ELSD) has also widened the applicability of HPLC for natural-product analysis [15]. This technique enables HPLC analysis of all nonvolatile compounds regardless of the absence or presence of a chromophore. Sophisticated coupling techniques like HPLC–mass spectrometry (MS), HPLC–nuclear magnetic resonance (NMR) spectrometry, and HPLC–solid-phase extraction (SPE)–NMR are increasingly used in the analysis

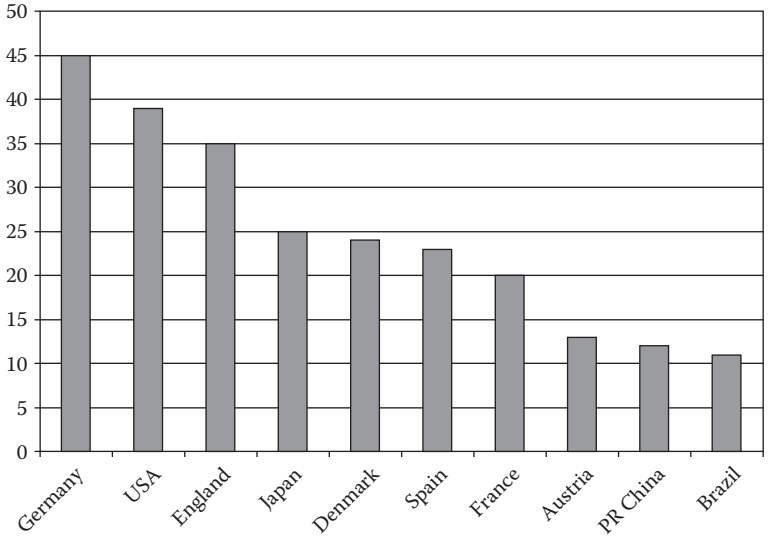

FIGURE 4.4 Countries contributing the most articles dealing with chemosystematics in the broadest sense (see terms listed in Figure 4.2) and HPLC from 1984 (first record) to 2008 based on the ISI Web of Knowledge database (accessed September 3, 2008).

of complex mixtures of natural products [16]. As neither data obtained from MS nor from NMR spectroscopy are useful in the determination of the absolute configuration of natural products, circular dichroism (CD) measurements combined with HPLC instruments provide an elegant means for the on-line determination of the absolute configuration of natural products [17]. Conclusively, nearly all techniques routinely used for structure elucidation and identification of natural products can be combined on-line with an HPLC apparatus. Besides its usage in quantifying particular compounds in complex mixtures, HPLC thus serves as a prerequisite for the fast and unambiguous determination of the structure of unknown compounds in complex mixtures.

4.3 HPLC-BASED CHEMOSYSTEMATIC STUDIES IN SYSTEMATIC BOTANY, MYCOLOGY, AND ZOOLOGY

4.3.1 Introduction

Initially perceived as the ideal way to elucidate phylogenetic relationships between species (mostly of plants), chemosystematics has lost much of its appeal to systematic botanists with the advent of molecular techniques enabling the investigation of DNA sequences [18]. However, in other groups of organisms like the archaea and bacteria, chemosystematics still has a leading role in the characterization and delimitation of new and established taxa.

In applied fields of botany like medicinal-plant research and research into the bioactive ingredients of plants used as foodstuffs, chemosystematic investigations are still of pronounced interest. In most investigations, only structurally identified compounds, that is, in those cases where particular peaks can be assigned to a particular known compound, are used as chemosystematic markers. However, comparative phytochemical investigations are also possible without any knowledge about the identity of the compounds responsible for the observed HPLC fingerprint patterns. One recent example of such an investigation is the differentiation between three morphologically similar *Baccharis* (Asteraceae) species by Lonni et al. [19]. Such studies are—similar to investigations using anonymous molecular markers—not easily reproduced, and their scientific impact is much lower than that of studies with properly assigned compounds.

An emerging field in chemosystematics is the combination of morphological, anatomical, chemosystematic, and molecular data. Molecular analyses sometimes result in the formation of unexpected clades, which are not easily aligned with traditional systems based on morphological characters. A prominent example is the family *Scrophulariaceae* s.l. The *Plantaginaceae* family in its newly expanded circumscription as well as the remaining *Scrophulariaceae* s.str. are morphologically not easily delimited from each other. In this as in many other less prominent examples, chemosystematic data provide an important alternative to morphological data in the phenetic characterization of phylogenetic lineages detected by molecular methods. Although the advantages of this combined approach are obvious, it is currently only sparsely used. The reason for this dissatisfying situation is probably a lack of communication between natural-product chemists and systematic (molecular) botanists.

4.3.2 Archaea and Bacteria

The number of bacteria species in the world is currently still unknown. Some authors estimate that the approximately 3000 species of archaea and bacteria that have been scientifically described constitute only 1% of the actually existing number of species [20]. Due to the minute size of bacteria and archaea, the applicability of morphological characters is limited, and the use of physiological and biochemical characters is traditionally of significant importance [21]. In combination with modern molecular techniques, chemosystematic and physiological investigations enable the unambiguous detection and characterization of bacterial species and strains [21]. This holds true not only for species that are already known but also for species new to science [22–29]. Kamekura and Kates showed that 12 of the 13 genera of the Halobacteriaceae, a family of archaea adapted to living in high salt environments, can be assigned to one or the other of the two known types of membrane-core lipids in the family [23]. The first group encompasses the genera *Halobacterium*, *Haloarcula*, *Haloferax*, *Halobaculum*, and *Halorubrum*. These genera contain only *sn*-2,3-diphytanylglycerol (Figure 4.5). In contrast, the genera *Halococcus*, *Haloterrigena*, *Natronobacterium*, *Natronococcus*, *Natronomonas*, *Natrialba*, and *Natrinema* contain both *sn*-2,3-diphytanylglycerol and *sn*-2-sesterpanyl-3-phytanylglycerol lipid cores (Figure 4.5). Moreover, some species of haloalkaliphiles also contain a *sn*-2,3-disesterpanylglycerol core lipid (Figure 4.5). A manuscript specifically dealing

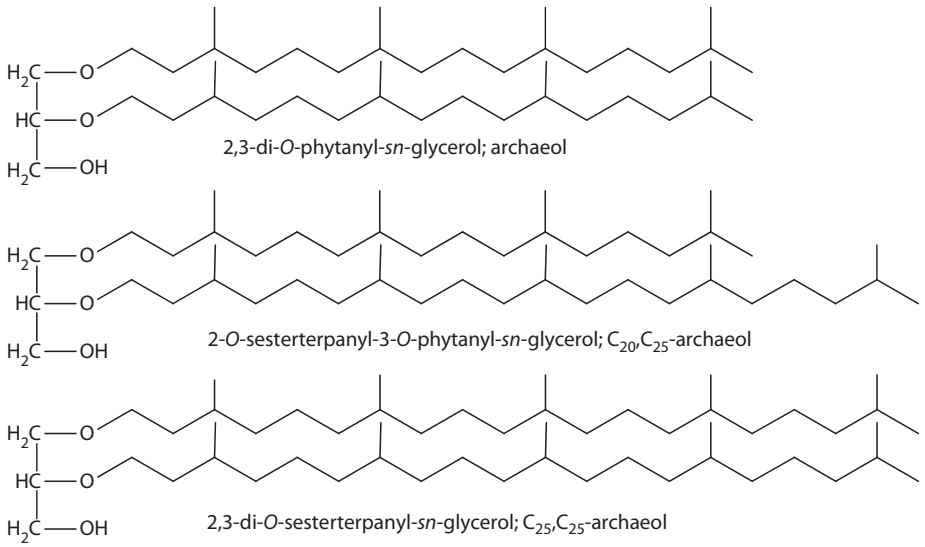

FIGURE 4.5 Core lipids of Halobacteriaceae, which provide chemosystematic markers for the characterization and delimitation of genera within this family of archaea.

with the methodology of HPLC–electrospray ionization (ESI)–MS identification of membrane lipids in archaebacteria was published by Qiu et al. [22].

The special role of chemosystematics in bacterial systematics is exemplified in the manuscript of Altenburger et al. [25]. In this paper a newly isolated bacterium species was assigned to a new genus, *Georgenia*, based on molecular data (16S rDNA). Besides the molecular characterization, the authors added data on the morphology, physiology, biochemistry, and chemosystematics of the new taxon. Chemosystematic data encompassed HPLC analyses of isoprenoid quinones, two-dimensional TLC investigations of polar lipids, and GC analyses of fatty acid methyl esters [25]. Indeed, the importance of chemosystematic methods in the characterization of new species of bacteria is so pronounced that some authors call for the inclusion of chemosystematic data in the formal description of new species of bacteria [30]. Besides techniques relying on HPLC separations and consecutive detection of the eluates with detectors such as UV, DAD, MS, or NMR, the direct investigation of bacteria with matrix assisted laser desorption ionization-time of flight (MALDI-TOF) MS is an elegant and fast approach to investigate the wealth of bacterial metabolites [31].

An in-depth account of the methods used in the systematics of bacteria was provided by Busse et al. [21]. In this review the following categories of chemosystematic data are listed: fatty acids, polar lipids, mycolic acids, liposaccharide composition, cell-wall diaminoacids, polyacrylamide gel electrophoresis (PAGE) of lipopolysaccharides, whole-cell sugars, cell-wall sugars, cellular pigments, quinone system, polyamine content, and whole-cell protein PAGE.

4.3.3 Animals

In animals, chemosystematic studies are less frequent than in microorganisms, plants, and fungi. Nonetheless, there are some systematic groups in which chemosystematic data are important to clarify borders between species and the phylogenetic relations between them. Termites are an interesting example. The soldier caste of these insects has frontal glands secreting species-specific defense chemicals. In European termites from the genus *Reticulitermes*, these secretions consist of monoterpenes, sesquiterpenes, diterpenes, and sesterterpenes [32]. For the analysis of mono- and sesquiterpenes, which also occur as regular components of plant essential oils, GC is usually the method of choice. However, in the case of *Reticulitermes*, HPLC is superior to GC because some of the excreted sesquiterpenes isomerize under GC conditions [32].

Chemosystematic studies in termites are not restricted to the analysis of the defense chemicals emitted by the frontal glands of the soldier caste but also encompass cuticular hydrocarbons of termites belonging to the worker caste [33]. Chemosystematic data on hydrocarbons in combination with molecular data on the mitochondrial haplotypes of the termites are well suited to determine species limits and to characterize morphologically ambiguous populations within the genus *Reticulitermes* [34]. A combination of workers' hydrocarbon and soldiers' defense-secretion data for North American termites revealed that there are more hydrocarbon than defense-secretion phenotypes and that each hydrocarbon phenotype is usually correlated to only one defense-secretion phenotype [35]. As morphological keys to *Reticulitermes* termites are inadequate to provide for unambiguous species delimitation and identification, Nelson et al. proposed to include chemical data in the description of species [35].

Species-specific hydrocarbon patterns were also observed in Australian canebeetles [36]. The similarity of compounds reflects the phylogeny of the beetles to some extent. Moreover, one particular sesterterpene seems to have a function in mate recognition in one of the species investigated [36].

Yet another example for chemosystematic investigations in the animal kingdom is the fatty acid composition of lipids in octocorals. An investigation of octocorals from the coastal waters of Vietnam revealed that the pattern of fatty acids by GC is a valid chemosystematic marker at the family level. Alyconaria, Antipatharia, Dendronephthya, and Gorgonaria formed well-separated clusters after principal-component analysis (PCA) of the fatty acid–quantification data [37].

4.3.4 Fungi

Chemosystematic methods have their place in both the systematics of macrofungi and microfungi. However, in the latter group chemosystematic data are much more important for the classification, delimitation, and identification of taxa. Examples for the application of chemosystematic data to the systematics of macrofungi include analyses of fatty acids in spores of higher basidiomycetes [38] and extrolites in members of the Xylariaceae [39,40].

There are many examples of chemosystematic studies in microfungi. Prominent examples include studies in *Penicillium* by Andersen et al. [41] and Overy et al. [42], in *Alternaria* and *Penicillium* by Hansen et al. [43], and in filamentous fungi in general by Frisvad et al. [44]. Andersen et al. provided an extensive study of the secondary-metabolite profiles of various accessions of *Penicillium expansum*, an airborne fungus that causes rot in many fruits, including apples, pears, and cherries [41]. The most problematic aspect of rotting fruit affected by these fungi is their ability to produce toxic metabolites, which are dominated by but not restricted to patulin. However, none of the strains investigated produced any of the particularly problematic compounds ochratoxin A, penitrem A, and rubratoxin B [41]. In their detailed study of *Penicillium* sect. Corymbifera, Overy et al. investigated multiple strains of each investigated species on various types of agar for their secondary-metabolite profiles [42]. The acquired data were then analyzed using modern methods of multivariate data analysis. From their extensive set of data, the authors constructed a diagnostic key for species determination taking phenotypic, chemotaxonomic, and pathogenic traits into account. Hansen et al. provided an approach that enables automated and unguided identification of various closely related *Alternaria* and *Penicillium* species solely based on their HPLC-DAD profiles [43]. The acquired classifications were in full accord with classifications achieved by classical methods. More detailed information on the field of microfungal chemosystematics is compiled in the review by Frisvad et al. [44]. This review gives a detailed account of the current state of knowledge and the methodologies employed.

A relatively recent field of research is the chemistry of marine fungi. One example is the study by Osterhage et al., who investigated the array of secondary metabolites occurring in marine and terrestrial representatives of the genus *Phoma* [45].

4.3.5 Phytoplankton

While chemosystematic analyses of higher plants are usually directed at the elucidation of relationships between the investigated taxa, employing secondary metabolites as the means, chemosystematic analyses of phytoplankton are an alternative technique for investigating the relative abundance of different classes of these microorganisms in a certain amount of water. In this field of science, accessory pigments and different types of chlorophyll, not secondary metabolites, are used to identify and quantify the various classes of microalgae present in the analyzed sample of (sea)water [46,47]. Prochlorophytes, diatoms, prymnesiophytes, pelagophytes, chrysophytes, cryptophytes, dinoflagellates, prasinophytes, and chlorophytes each feature a characteristic set of chlorophylls and carotenoid pigments [47]. Detailed up-to-date information on the various chlorophylls known from picophytoplankton was compiled by Zapata [48].

Algorithms have been established that allow for the translation of the observed HPLC peaks and their areas into the absolute and relative contributions of various classes of algae to the phytoplankton in a given amount of sea water. In most instances the results obtained are well in line with the results of classical electron-microscopic investigations of phytoplankton in seawater. The advantage of HPLC is the short analysis time, requiring less expert knowledge than the interpretation of electron-microscopic data [47].

HPLC analyses of phytoplankton composition allow the determination of the taxonomic spectrum and relative abundance of microalgae in different regions, seasons, and years as well as their change under differing ecological conditions [49]. The high interest in using chemosystematic methods for

the analysis of phytoplankton composition is indirectly mirrored in the high number of citations (259 citations) one of the basic methodological papers by Mackey et al. has received up to the present day (ISI search performed on November 24, 2008) [50].

Considerable effort has been and is being put into the amendment of the existing protocols for sample preparation and extraction [51,52], HPLC methodology [53–55], and data evaluation [47,50]. In conclusion, pigment analysis by HPLC is an important tool in the study of phytoplankton field samples from open-ocean and coastal waters [48]. Alternative and complementary techniques include microscopic, electron-microscopic, and molecular techniques [47–49].

4.3.6 Lichen Metabolites

The classical paper on the HPLC analysis of lichen metabolites was published by Feige and coworkers [56]. In this paper, retention indices of 331 of the approximately 450 aromatic lichen metabolites known then were reported. As lichens are hard to determine with morphological characteristics alone, and as color reactions with chemical agents have a long tradition in lichenology, chemosystematic data for lichens are more abundant than for most other groups of organisms. A user-friendly protocol for how to collect chemosystematic data from lichens using both TLC and HPLC was provided by Lumbsch [57]. The class of compounds predominantly used as chemosystematic markers in lichens are lichen acids, a unique class of phenolic compounds that is biosynthesized only by lichens [56–58]. An exception to this rule is the account by Sassaki et al. [59]. These authors analyzed the fatty acid composition of lichenized fungi. Hydrolized fatty acids were separated by partitioning and detected by GC-MS as their methyl esters after esterification with methanol. A highly innovative approach for lichen chemosystematics was presented by Alcantara et al. [60]. Proton high-resolution magic-angle-spinning NMR (^{1}H HR-MAS NMR) and Fourier transform infrared (FT-IR) data were used as alternative means to discriminate between 11 selected lichen species belonging to six different genera and two families. After acquisition the data obtained were analyzed by multivariate data-analysis techniques (PCA and hierarchical-cluster analysis). The groupings obtained with this approach mostly coincided with the taxonomic groupings of the analyzed lichens, and the discriminatory power was comparable to classical HPLC quantification data. This new approach is nondestructive and requires no elaborate sample preparation. A wide application of the presented methods to groups other than lichens will, however, be hindered by the fact that the obtained signals are mostly derived from interactions with the lichens' carbohydrates. Thus, for higher plants with their rather uniform pattern of main carbohydrates in the intact plant, little interspecific variation is to be expected.

4.3.7 Higher Plants

4.3.7.1 Alkaloids

Alkaloids are a fascinating class of natural products for many reasons. Alkaloids encompass an amazing diversity of chemical structures, usually containing one or more nitrogen atoms included in a variety of different ring systems. Many alkaloids have exceptional bioactivities, like morphine (a drug for pain relief), coniine (a deadly poison), or caffeine (a stimulant). Alkaloids also provide a very interesting class of marker compounds for chemosystematic studies. However, some alkaloids have an erratic distribution in the plant kingdom. Such a pattern often implies parallel evolution in the plants' ability to synthesize this particular compound.

One example of an erratically distributed alkaloid is caffeine. The ability to biosynthesize caffeine, theobromine, and/or theophylline was probably independently acquired by a number of groups that are not closely related phylogenetically. Caffeine is a well-known ingredient of taxa from the genera *Camellia* (tea, Theaceae), *Coffea* (coffee, Rubiaceae), *Cola* (cola, Sterculiaceae), *Erythroxylum* (coca, Erythroxylaceae), *Ilex* (mate, Aquifoliaceae), *Paullinia* (guarana, Sapindaceae), *Sterculia* (tropical chestnut, Sterculiaceae), and *Theobroma* (cacao, Sterculiaceae) [61,62]. However, caffeine

also occurs in the plant genus *Sinomenium* (Menispermaceae) [63], the fungal genus *Claviceps* [64], and even in the gorgonian genus *Villagorgia* [65]. This distribution pattern, which includes animals, fungi, and higher plants, implies that the ability to synthesize caffeine was either acquired early in evolution and subsequently lost by most species or independently acquired by different unrelated phylogenetic lineages in animal, fungi, and plants. Caffeine and the related compounds theobromine and theophylline are derived from the same pathway as the purine bases adenine and guanine. Therefore, the starting material for the biosynthesis of caffeine is rather ubiquitous, and the ability to biosynthesize caffeine was probably acquired independently in disparate groups of living organisms. As a result, caffeine and the related compounds theobromine and theophylline are not suitable as chemosystematic markers at higher levels of the systematic hierarchy. Interestingly, caffeine is sequestered from the tea plant [*Camellia sinensis* (L.) Kuntze] by its parasite *Scurrula atropurpurea* (Blume) Danser (Loranthaceae) [66].

In contrast to the purine alkaloids and their erratic distribution, other classes of alkaloids are restricted to one or few systematic groups and are valuable systematic markers in the delimitation of these groups. One example of a chemosystematically interesting alkaloid is colchicine. This highly toxic compound, which is medicinally used in the treatment of acute gout and is also used in plant breeding to obtain polyploid plants, is restricted in its distribution to the Colchicaceae family. The Colchicaceae, formerly part of the Liliaceae family, are now a family within the Liliales order and comprise 245 species in 22 genera: *Androcymbium*, *Baeometra*, *Bulbocodium*, *Burchardia*, *Camptorrhiza*, *Colchicum*, *Disporum*, *Gloriosa*, *Hexacyrtis*, *Iphigenia*, *Kuntheria*, *Littonia*, *Merendera*, *Neodregea*, *Onixotis*, *Ornithoglossum*, *Petermannia*, *Sandersonia*, *Schelhammeria*, *Tripladenia* (including *Kreysigia*), *Uvularia*, and *Wurmbea* [67,68]. According to the SciFinder and MDL CrossFireCommander databases (both accessed on October 15, 2008), the alkaloid colchicine has been detected in or isolated from members of the following genera (nomenclature updated to current usage in some cases): *Androcymbium*, *Bulbocodium*, *Camptorrhiza*, *Colchicum*, *Gloriosa*, *Iphigenia*, *Littonia*, *Merendera*, *Ornithoglossum*, *Sandersonia*, *Tripladenia*, and *Wurmbea*. Thus, colchicine is known from more than 50% of the genera of the Colchicaceae family but has never been detected in a taxon from any other family. At this stage colchicine is an excellent chemosystematic marker for the Colchicaceae family, and it is reasonable to assume that chemosystematic investigations of the genera of the Colchicaceae that are not yet known to contain colchicine will reveal additional sources of this toxic and medicinally important natural product [61].

A second example of an alkaloid that provides valuable chemosystematic information is protopine. Protopine is characteristic for the Papaveraceae family s.l. (i.e., including the Fumarioideae). The MDL CrossFireCommander database lists the following genera as sources of protopine (in alphabetical order; familial affiliation is indicated in parentheses): *Argemone* (Papaveraceae), *Adlumia* (Papaveraceae), *Bocconia* (Papaveraceae), *Caltha* (Ranunculaceae), *Ceratocapnos* (Papaveraceae), *Chelidonium* (Papaveraceae), *Corydalis* (Papaveraceae), *Dactylicapnos* (Papaveraceae), *Dicentra* (Papaveraceae), *Eschscholtzia* (Papaveraceae), *Fumaria* (Papaveraceae), *Glaucium* (Papaveraceae), *Hylomecon* (Papaveraceae), *Hypecoum* (Papaveraceae), *Macleaya* (Papaveraceae), *Meconopsis* (Papaveraceae), *Nandina* (Berberidaceae), *Papaver* (Papaveraceae), *Platycapnos* (Papaveraceae), *Platystemon* (Papaveraceae), *Roemeria* (Papaveraceae), *Romneya* (Papaveraceae), *Sanguinaria* (Papaveraceae), *Sarcocapnos* (Papaveraceae), *Stylomecon* (Papaveraceae), *Stylophorum* (Papaveraceae), *Thalictrum* (Ranunculaceae), and *Zanthoxylum* (syn., *Fagara*, Rutaceae). Thus, with four exceptions all reported sources are members of the Papaveraceae s.l. When checking the nonpapaveraceous sources in detail, the report from *Fagara*/*Zanthoxylum* turned out to be a wrong entry into the database. Fish and Waterman actually never reported protopine from *Fagara* but just one of its derivatives [69]. The other nonpapaveraceous sources of protopine are *Caltha* [70], *Nandina* [71], and *Thalictrum* [72]. According to Angiosperm Phylogeny Group II all three species are members of the same order as the Papaveraceae, the Ranunculales order [67].

The preceding examples highlight that, depending on the particular alkaloid and the complexity of its biosynthesis, alkaloids can be either very poor or very reliable chemosystematic markers.

4.3.7.2 Flavonoids and Related Compounds

Flavonoids have been detected in nearly all species of higher plants where their occurrence has been investigated. This, together with the fact that they are easily detected using even simple techniques like PC and TLC, made flavonoids the best-investigated group of plant secondary metabolites in the early days of chemosystematic research. The analysis of flavonoids is particularly suited to demonstrate the technological advances in chemosystematic research from the 1940s to the present day. Bate-Smith published a then-pioneering paper in *Nature* on the use of two-dimensional PC for the detection (and chemosystematic interpretation) of anthocyanins and related compounds in 1948 [4]. PC was soon widely replaced by TLC in most applications [73].

HPLC was first used in the analysis of flavonoids in the early 1970s [8]. The next step was the development of the on-line coupling of HPLC with a mass spectrometer [74]. Around the same time the first steps were taken to make on-line HPLC-NMR possible [75]. Applications in flavonoid research followed [76]. However, only since the additional development of SPE systems can the cost of deuterated solvents be kept at a reasonable level, and now the quality and reproducibility of spectra reaches the same level as those of traditional off-line acquired spectra [77].

4.3.7.3 Iridoids

Iridoids are known from various orders of higher plants, for example, Dipsacales, Ericales, Gentianales, and Lamiales. The occurrence of particular subgroups and substitution patterns of iridoids has been successfully exploited in a number of chemosystematic studies. Before some of these studies are presented in more detail, a prominent misplacement, which was deemed to be supported by chemosystematic data, will be mentioned. Based on morphological characteristics, the Menyanthaceae was traditionally regarded either as a subfamily of the Gentianaceae [78], as a separate family that was part of the Gentianales order [79], or, alternatively, as a separate family that was a member of the Solanales order [80]. The affinity of the Menyanthaceae to the Gentianales was supposedly backed up by secondary-metabolite chemistry. Both the Gentianaceae and the Menyanthaceae contain secoridoids, a class of compounds notable for their extreme bitter taste. This is used in medicine in the form of *Amara*, bitter preparations used to induce appetite. To this end, members of both families, such as *Gentiana lutea* L. from the Gentianaceae and *Menyanthes trifoliata* L. from the Menyanthaceae, are used in phytomedicine. However, with the advent of molecular techniques in systematic botany, the Menyanthaceae had to be unexpectedly transferred to the Asterales order [81,82]. Chemical evidence that the Menyanthaceae were actually misplaced in the Gentianales was earlier provided by Bohm et al. [83], who analyzed the flavonoid chemistry of both families, and, more important, by Pollard and Amuti [84], who discovered that taxa belonging to the Menyanthaceae—like taxa of the Asteraceae—employ inulin as their reserve carbohydrate and not starch as do most other higher plants. This clearly indicates that in higher plants, as in algae, biochemical characteristics derived from compounds involved in or close to the primary metabolism are more important than secondary metabolites.

Other groups that have been systematically investigated for the occurrence of iridoids and their chemosystematic potential include the genus *Lamium* (Lamiacaeae), the Loasaceae family, the Oleaceae family, the genus *Plantago* (Plantaginaceae), and the tribe Veroniceae (Plantaginaceae). Similar to the situation in sesquiterpene lactones, chemosystematic studies using iridoids as chemosystematic markers are often based on the actual isolation of the compounds from the studied species. One notable exception is the account of Alipieva et al., who studied the occurrence of nine iridoids in five species of the genus *Lamium* using HPLC-ESI-MS [85]. Using a combined approach of TLC and HPLC, Müller et al. screened 74 species of the Loasaceae subfamily Loasoideae for the occurrence of 13 iridoids, 11 of which were structurally identified [86]. Unfortunately, these authors did not publish analytical details (retention times and retention factors) of all their analytes.

In an extensive study Rønsted et al. isolated a total of 35 iridoids from 34 species of *Plantago* s.l [87]. Most compounds were isolated from multiple sources, and the distribution of the particular

compounds was analyzed in comparison to systematic groupings within the genus. Iridoids proved to be valuable chemosystematic markers for the delimitation of intrageneric entities. The same approach was chosen to investigate 14 members of the Veroniceae tribe of the Plantaginaceae family [88]. Again compounds were isolated, their structure elucidated by NMR and MS, and the obtained results regarding the distribution of the compounds compared to existing molecular groupings of the analyzed systematic group. Here, as in the previous example and in an extensive review of iridoids and their distribution in the Oleaceae family, the natural-product class of iridoids proved to be very useful to phenetically characterize groupings emerging from molecular studies [89]. The approach of individually isolating the compounds used as chemosystematic markers is by far the most probative way to prove the occurrence of a particular compound in a particular source species. However, it is also the most time- and labor-consuming way. Many TLC-based studies from the early days of chemosystematics lacked a high degree of certainty in the identification of chemosystematic markers, because only retention factors that were hard to reproduce and color reactions were employed for compound identification. The better resolution achievable with HPLC made peak/compound assignments safer, and HPLC-MS is nowadays usually regarded as an adequate way to prove the existence of a particular compound in a given source. However, as this approach also depends on a good HPLC separation, this dictum has to be tested in each particular case. The existence of many isomers with similar HPLC retention times in some instances requires either more elaborate on-line detection systems (like HPLC-NMR) or the isolation and subsequent off-line structure elucidation of the compound at hand to finally prove its occurrence in the investigated species.

4.3.7.4 Sesquiterpene Lactones

Sesquiterpene lactones are secondary plant metabolites that predominantly occur in the Asteraceae family. Other sources include such diverse groups as the liverworts and the Apiaceae, Lamiaceae, Lauraceae, and Magnoliaceae families [62]. Sesquiterpene lactones comprise the active principles of many medicinal plants from the Asteraceae and therefore have been studied extensively. Moreover, the various classes of sesquiterpene lactones occurring in the Asteraceae have a pattern of distribution that is in good congruence with the infrafamilial systematics of the family [90]. Though sesquiterpene lactones have extensively been evaluated as chemosystematic markers, there are comparatively few papers dealing with HPLC and chemosystematics employing sesquiterpene lactones as markers. Most papers on sesquiterpene lactones describe the isolation and structure elucidation as well as the bioactivity of sesquiterpene lactones from one particular source. Chemosystematic interpretations are then usually made in reviews compiling the information from a number of such papers assessing the structure elucidation and bioactivity. Nonetheless, HPLC is an excellent means of analyzing the distribution of sesquiterpene lactones in the Asteraceae family, and some papers explicitly using HPLC for sesquiterpene lactone-based chemosystematics are discussed in the following.

Zidorn et al. described the HPLC separation and on-line MS data of all 13 currently known members of the hypocretenolide subclass of guaiane-type sesquiterpene lactones [91]. Hypocretenolides feature an unusual 12,5-lactone ring and have so far been detected exclusively in the Lactuceae tribe of the Asteraceae family. Using a similar system, Zidorn et al. described HPLC separation and on-line MS data of six 12,6-guaianolides and their distribution within the genus *Leontodon* s.l. [92].

Spring and Schilling analyzed 18 taxa of *Helianthus* (Asteraceae) for the occurrence of sesquiterpene lactones [93]. From the 43 compounds tentatively assigned as sesquiterpene lactones, the structures of 21 were identified with the help of reference compounds. With the exception of one species, all investigated *Helianthus* taxa contained a number of sesquiterpene lactones. The occurrence of the known compounds within the genus, together with structural similarities between the detected compounds, was employed to assign the taxa investigated to three well-defined groups.

HPLC analysis of *Helianthus annuus* L. and *H. debilis* Nutt. as well as of both possible hybrid combinations (*H. annuus* x *H. debilis* and *H. debilis* x *H. annuus*) revealed that hybrids' additive chemical character traits, which are well documented for flavonoid patterns, are also observable in

the hybrids' sesquiterpene lactone patterns [94]. Moreover, this study showed that besides additive sets of sesquiterpene lactones derived from both parent species, hybrids contained a number of additional chimer-compounds. These compounds consisted of a basic sesquiterpene lactone found in one parent and an additional side chain found in metabolites from the other parent.

Endemic species of *Scalesia* from the Galapagos Islands were analyzed by HPLC-MS and HPLC-NMR for their patterns of flavanones and sesquiterpene lactones [91]. The sesquiterpene lactone—screening part of this study was possible with very limited plant material due to the application of a microextraction technique focused solely on glandular trichomes, which were particularly rich in sesquiterpene lactones.

4.4 HPLC-BASED CHEMOSYSTEMATIC STUDIES IN FOOD SCIENCE

4.4.1 Introduction

Reynolds states in the first two sentences of his account on the evolution of chemosystematics the following fundamental dictum: "Chemosystematics has been used to distinguish plants and other organisms that are useful for food and those best avoided. Originally unwritten, this knowledge has been progressively formalized with useful, harmful, and inactive chemical constituents from relevant taxa now identified and recorded" [96].

Though chemosystematic knowledge was paramount for the survival of human societies from the earliest times, the term *chemosystematics* is rarely if ever mentioned in modern accounts of food chemistry. There are, however, numerous papers dealing with the distribution of "useful, harmful, and inactive chemical constituents" in foodstuffs. These data are rarely assessed for their possible use as systematic markers in botany though they have a lot of potential in this field. These data are, however, surveyed and analyzed to assess the quality of food, to verify the origin of food with regards to the botanical or zoological species the food is derived from, and to verify the geographic origin of the investigated samples. The following three sections highlight the significance of chemosystematics in food science with three examples. The first example, honey, is a food only indirectly derived from plants. Nevertheless, the chemical composition of honey, including its sugars and plant secondary metabolites, is an important marker to reveal the source plant species the honey was derived from. This is of special importance for filtered honeys. In filtered honeys the classical approach to verify honey identity, pollen analysis, is not implementable because the characteristic pollen grains are removed from the honey during filtration. The second example deals with olive oil. In olive oil the source species, the olive tree *Olea europaea* L., is undisputed unless the oil is illegally adulterated. However, the quality of the oil, including varietal identity, manufacturing process, and geographic origin, is mirrored in its chemical composition. The third example is coffee. This extremely important tropical agricultural crop is derived from various species of the genus *Coffea* (Rubiaceae). Besides the identity of the source species, the region of origin, ecological factors of the growing site, and the manufacturing process influence the chemical composition of the end product, coffee.

4.4.2 Unifloral Honeys

The floral origin of honey is its identity criterion and one of its main quality parameters. In general, unifloral honeys achieve higher prices on the market than multifloral honeys, a fact that tempts some producers and vendors to mislabel their products with regards to the source of their honeys [97]. According to the European Council directive 2001/110/EC on honey, the blending of honeys from different geographic or floral origins is permitted provided products are labeled correctly [98]. Traditionally, the floral origin of honey is assessed by pollen analysis, a technique also referred to as melissopalynology. This method, although undemanding with regards to instrumentation, has some shortcomings, among them the dependence on highly trained specialists and the need of a

complete pollen library [99]. Furthermore, pollen analysis is time consuming, and unifloral nectar may be contaminated with foreign pollen due to flower morphology, even in so-called unifloral honeys. Pollen profiles might be misleading due to overrepresentation (for example, in *Castanea*, *Eucalyptus*) or underrepresentation (for example, in *Arbutus*, *Citrus*, or *Rosmarinus*) of certain nectar source species. Besides interspecific differences in the numbers of pollen grains produced, flower morphology (for example, *Arbutus* flowers are in an upside-down position) affects the extent of nectar contamination with pollen [100]. In honeys derived from sterile plants, pollen analysis is obviously not suited to guarantee identity [101]. The European Council directive on honey prohibits pollen from being removed from honey during processing. Exceptions are permissible if organic or inorganic foreign matter needs to be removed, for example, via filtration or centrifugation [98]. Here, pollen analysis is also not the appropriate tool to assess honey's botanical origin.

In recent years, there has been an increasing interest in establishing analytical methods complementary to pollen analysis in attempts to verify honey authenticity with regards to its floral and geographic origin [102–105]. These alternatives include measurements of physicochemical properties such as pH, electrical conductivity, water content, and color [106,107], identification of specific chemical markers like particular carbohydrates [97,108–110], volatile organic compounds [99,111–114], and phenolics [100,115–117]. Many of these investigations are based on quantitative differences in the analyzed compounds. Data analysis then requires multivariate statistical-pattern-recognition methods for the identification of honeys derived from different origins.

The main components of honey are glucose and fructose, usually comprising 65–80% of the total soluble solids and comprising 85–95% of honey's carbohydrates [108]. Honey oligosaccharides may originate from either nectar, honeydew, or α-D-glucosidase activity. This enzyme is contained in the saliva of honeybees and catalyzes the transfer of α-D-glucopyranosyl groups from sucrose to acceptor carbohydrates [110]. Variable amounts of sucrose, glucose, and fructose found in nectars of different floral origins indicate that honey sugar profiles may potentially be employed as chemotaxonomic fingerprints [97]. Carbohydrate profiles of honeys have been analyzed by various methods. Swallow and Low separated 20 structurally similar carbohydrates in four honeys of known botanical origin (alfalfa: *Medicago sativa* L. subsp. *sativa*; alsike: *Trifolium hybridum* L.; canola: *Brassica napus* L.; and trefoil: *Lotus corniculatus* L.), employing high performance anion-exchange liquid chromatography with pulsed amperometric detection [108]. The major carbohydrates in honeys derived from all four source species were identical and present in similar amounts: glucose (*Medicago* 33.9%, *Trifolium* 35.6%, *Brassica* 40.0%, *Lotus* 33.2%), fructose (*Medicago* 37.0%, *Trifolium* 38.5%, *Brassica* 36.2%, *Lotus* 37.1%), and maltose (*Medicago* 1.18%, *Trifolium* 1.00%, *Brassica* 0.76%, *Lotus* 1.11%). In contrast, sucrose was present in the leguminous taxa *Medicago* (1.18%), *Trifolium* (0.90%), and *Lotus* (0.55%) in similar but low amounts. In the cruciferous taxon *Brassica*, this sugar was detectable as a trace compound only (0.046%). Similarly, erlose is present in significant amounts (>2.5%) only in the Fabaceae species but present in traces only in *Brassica* (<0.5%). In conclusion, oligosaccharide patterns might be used to differentiate honeys from leguminous source species from those from cruciferous source species. More relevantly, carbohydrate patterns may be employed in the search for honeys illegally adulterated with invert sugar or corn syrup.

Based on Swallow and Low's method [108], Goodall et al. analyzed 40 carbohydrates in six unifloral honeys: bramble—*Rubus fruticosus* L. s.l.; ling (heather)—*Calluna vulgaris* (L.) Hull; oil seed rape—*Brassica napus*; white clover—*Trifolium repens* L.; hawthorn—*Crataegus* div. spec., mainly *C. monogyna* Jacq.; and willow herb—*Epilobium* div. spec. [97]. Consecutively, quantitation results were subjected to canonical discriminant analysis. The applied chemometric method allowed for correct classification of 100% of the ling samples, 70% of the bramble samples, and 63% of the oil seed rape samples, but none of the white clover samples was assigned correctly. The reason for the misclassification of white clover samples was reasoned to be due either to the small data set or to contamination of the white clover samples with bramble. This contamination was verified by pollen analysis in the order of 21 and 25%, respectively, for two of three samples investigated.

Probably because of the unbalanced number of samples per species (many more bramble samples were analyzed than from any of the other source species) and the limited overall number of samples available, discrimination of hawthorn and willow herb samples was not clear cut.

Rufino and Bosch-Reig assessed the contents of 15 sugars by GC of their silyloxime derivatives in seven Spanish unifloral honeys (rosemary—*Rosmarinus officinalis* L.; orange blossom—*Citrus* div. spec.; lavender—*Lavandula latifolia* Medik.; sunflower—*Helianthus annuus*; eucalyptus—*Eucalyptus camaldulensis* Dehnh. and *E. globulus* Labill.; various taxa from the Ericaceae; and honeydew honey from *Quercus* div. spec. [109]. Discriminant analysis revealed that fructose, glucose, sucrose, maltose, and the glucose/water ratio were best suited to assign honeys to different sources. Employing this methodology, 100% of honeydew, 92.9% of sunflower, 83.3% of heather, and 75.0% of eucalyptus honey samples were assigned correctly. Correct classification of the other honey samples was below 70%. Other sugar parameters proved to be useful to discriminate between just two different unifloral honeys or to distinguish one honey from all other honeys. For example, kojibiose allowed for separation of rosemary and orange flower honey samples, and orange flower honeys were best differentiated from all other honey samples due to their relatively high sucrose content.

Another class of chemicals frequently employed in chemosystematic studies of honey are phenolic acids and flavonoids. The main source of flavonoids in honey is neither pollen nor nectar but propolis. Propolis is a sticky resin collected by bees from several plant species and used, for example, in the construction of the beehive and to embalm dead invaders too large to be removed from the beehive [102]. Numerous compounds have been detected in propolis, among them flavonoids, benzoic acid derivatives, benzaldehyde derivatives, cinnamic acid derivatives, and terpene derivatives [118]. Propolis flavonoids most commonly found in honeys are the flavanones pinocembrin and pinobanksin, the flavones chrysin and galangin, and methyl ethers of the flavonols quercetin and kaempferol. The extremely lipophilic flavone tectochrysin (Figure 4.6) is also a constituent of propolis and when present in honey indicates contamination of the honey with beeswax [119]. The significance of propolis-derived phenolics as chemotaxonomic markers is modest. In contrast, the varying amounts of particular flavonoids from the nectar and pollen of various plant species, like quercetin, kaempferol, luteolin, 8-methoxykaempferol, apigenin, and isorhamnetin, are good indicators for the botanical origin of honey [119]. For example, the main source of the aglycon kaempferol in rosemary honey is kaempferol 3-*O*-sophoroside, which is present in high amounts in rosemary nectar [102]. Hesperetin has until now been detected only in citrus honey and is also present in the nectar and pollen of citrus flowers [120]. In a study carried out by Andrade et al., capillary-zone electrophoresis was employed to determine characteristic phenolic compounds in honeys of different floral origins, among them several phenolic acids [116]. According to this investigation, rosmarinic acid is characteristic for thyme (*Thymus capitatus* Hoffmanns. & Link) honey, naringenin and *m*-coumaric acid for lavender (*Lavandula stoechas* L.) honey, and ellagic acid and phenylethyl caffeate for heather (*Erica* div. spec.)–derived honey. Homogentisic acid was detected by HPLC and found to be suitable as a marker for strawberry-tree (*Arbutus unedo* L.) honey [100].

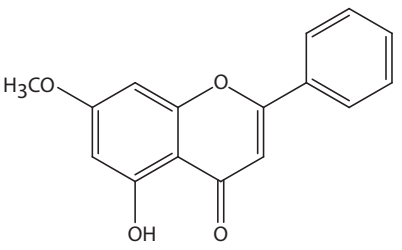

FIGURE 4.6 Structure of tectochrysin, an apolar flavonoid regularly found in propolis and propolis-contaminated honey.

Many recent studies dedicated to the determination of the botanical origin of honey focused on volatile organic compounds extracted by various techniques, such as SPE, solid-phase microextraction (SPME), liquid–liquid extraction, hydrodistillation, and simultaneous-distillation extraction (SDE). Volatiles are usually analyzed by methods other than HPLC, such as GC, GC-MS, and GC-olfactory analyses. These volatiles, too, are promising chemotaxonomic markers for the assessment of the botanical origin of honey [99,121–123].

4.4.3 Olive Oil

Olive oil is extracted from the mesocarp of *Olea europaea* (Oleaceae) fruits. Due to its fine taste and generally assumed health-promoting properties, olive oil obtains higher prices on the market than most other vegetable fats, such as those from soy, hazelnut, or sunflower [124]. The majority of olive oils sold throughout the European Community are blends of oils from different geographic origins [125]. As long as the character and origin of the oil are clearly specified on the label, it is not illegal to sell olive oil blended with either olive oil from different regions or other vegetable oils. However, keeping in mind that high market prices are achieved mainly for olive oils of the extra virgin and virgin categories, it is obvious that these are the categories mainly subjected to adulteration and mislabeling. However, mislabeling occurs not only with regards to the oil's category but also with regards to the geographic and cultivar origin.

Thus, it is essential to establish methods sensitive enough to detect adulterations of olive oil with other, economically inferior oils. Other important quality criteria for olive oil are geographic origin and cultivar. The composition of olive oil is influenced by factors like temperature, precipitation, soil, and altitude [126].

Routine testing of edible oil and fat authenticity focuses mainly on the lipophilic fraction. Triacylglycerols, the main apolar components in vegetable oils, are useful chemosystematic markers to detect admixing of foreign oils with olive oil. The European Commission Regulation (EEC) No. 2568/91—last amended by Commission Regulation (EC) No. 640/2008 of July 4, 2008—demands the application of an HPLC method to detect adulterations with canola, sunflower, and soybean oil [127,128]. Based on this method olive oil containing more than 0.5% of trilinoleate and dilinoleyl-linoleate is considered to be adulterated, because both compounds are present in olive oil in traces only. Adulterations of olive oil with one or more of eight different vegetable oils (avocado, almond, grape seed, linseed, mustard seed, olive, pumpkin seed, sesame seed, and soybean) are detectable employing the method described by Jakab et al. [129]. In this study, the triacylglycerol profiles of different vegetable oils were assessed by HPLC-APCI-MS. Linear discriminant analysis allowed for a 100% correct classificiation of monovarietal samples of these oils. However, it has not been assessed to which degree these oils may be detected when mixed with each other.

Besides the triacylgylcerols, other lipophilic compounds are useful chemical markers for testing oil authenticity. Oils rich in linoleic acid, such as sunflower, soy, and corn oil, are detectable in olive oil by HPLC even in quantities less than 1% [130]. Tocotrienol is useful to detect small amounts of palm and grape seed oils (1–2%) in olive and other tocotrienol-free oils by HPLC [131]. Koprivnjak et al. were able to correctly identify olive oil samples obtained from three different cultivars (Leccino, Buza, and Bjelica) grown in Croatia by LC-MS analysis of their *n*-alkane profiles [132]. Although samples were obtained from olives collected in different harvesting periods and years, discrimination was successful in > 95% of the investigated samples. Nagy et al. successfully distinguished oil samples from three olive varieties (Nocellara, Biancolilla, and Cerausola) as well as mixtures of two of these varieties based on their sitosterol, γ-tocotrienol, and tri- and diacylglycerol contents [133]. Olive oils extracted from the fruits of four different cultivars (Coratina, Leccino, Oliarola, and Peranzana) were discriminated employing ^{13}C-NMR spectra of fatty acids, sterols, and triacylglyderides by Brescia et al. [134]. Cañabate-Díaz et al. distinguished different olive oil-manufacturing categories (virgin, refined, olive-pomace, and crude olive-pomace oil) by means of their sterol content assessed with LC-MS [135].

An agent frequently used to adulterate olive oil is hazelnut oil. Hazelnut and olive oil exhibit a similar chemical profile. Hazelnut oil contains the characteristic volatile lipophilic compound (*E*)-5-methylhept-2-en-4-one (filbertone), which allows for the unambiguous identification of hazelnut oil. However, when hazelnut oil is mixed with olive oil, filbertone is hard to detect due to the dilution, thus limiting the traceability of hazelnut oil. Moreover, filbertone is partially lost during the oil-refining process. Therefore, detection of the adulteration of olive oil with refined hazelnut oil is sometimes problematic. Flores et al. detected adulterations of olive with virgin and refined hazelnut oil in amounts of 5% and 10%, respectively, by an on-line coupling of HPLC and GC [136].

An alternative approach to detecting hazelnut oil in olive oil was chosen by Zabaras and Gordon, who investigated the polar fraction of olive oil [137]. These authors compared the nonvolatile phenolic profiles of olive oil mixed with different amounts of hazelnut oil by means of HPLC. Detection of adulterations with hazelnut oil down to a level of 5% was successful in 80% of the investigated adulterated olive oil samples. However, this method is not as specific as the one proposed by Flores et al., since phenolic profiles are variable depending on the origins of both the hazelnut and olive oils [136].

Andjelkovic et al. investigated the phenolic profiles of five different olive cultivars from France and Spain [138]. The total phenolic content was assessed with the Folin-Ciocalteu method, and particular phenolic compounds were analyzed with LC-MS. Compared to the Spanish samples, the French samples exhibited lower total amounts of phenolics. All cultivars were distinguished by their α-tocopherol contents. In the French samples, a correlation between the luteolin content and the altitude of the growing site was observed.

In a comprehensive review article, Arvanitoyannis and Vlachos summarized a range of studies implementing physicochemical, sensory und multivariate statistical analyses in olive oil quality assessment [139]. Among numerous analytical methods employed to successfully detect the geographic origin and/or adulterations of olive oil with other vegetable oils are GC, GC-MS, and GC-stable isotope ratio mass spectrometer (IRMS); SPME followed by multidimensional GC [140,141]; and NMR-based methods, such as distortionless enhancement by polarization transfer (DEPT) [142] and ^{13}C-, ^{1}H-, and ^{31}P-NMR [143–145]. Moreover, Fourier transformed mid-infrared (FT-IR), near-infrared (FT-NIR), and Raman (FT-Raman) spectroscopy [146] as well as total luminescence and synchronous scanning fluorescence spectroscopy [147], carbon isotope discrimination [148], and spectrometry based on X-ray scattering were used in olive oil authentication. Many of these methods allow the reliable classification of selected olive cultivars, the correct geographic determination, a satisfactory discrimination of different olive oil-manufacturing categories, and/or the identification of the most frequently occurring adulterations [149].

4.4.4 Coffee

Coffee is a beverage prepared from the roasted and ground seeds, commonly called "beans," of plant species from the genus *Coffea* (Rubiaceae). Two coffee species, *C. arabica* L. (referred to as arabica) and *C. canephora* Pierre ex Froehn. (referred to as robusta), account for 99% of the total world coffee production [150]. Compared to robusta, arabica coffee has a finer and more pronounced flavor. Arabica seeds develop and ripen more slowly than robusta seeds, resulting in lower yields of arabica. Coffee quality is not affected only by the variety but also by geographic and environmental factors [151]. However, generally, arabica coffee attains 2–10 times the market price of robusta coffee [152]. Therefore, the confirmation of coffee varietal authenticity is of considerable economic interest. While it is easily feasible for coffee experts to distinguish whole green arabica from robusta beans visually, types of roasted and ground coffee are not easily discerned. Thus, alternative strategies such as discrimination by means of chemical or physical parameters are warranted. Among the chemical compounds used as descriptors for coffee samples are fatty acids [153], amino acids [150], volatile organic compounds [154,155], diterpenes [156], caffeoyl quinic acid derivatives [157,158], elements [159], and tocopherols [152,160], as well as particular compounds such as trigonelline,

caffeine, and nicotinic acid [158,161]. In numerous investigations, analyses of organic compounds were performed either by HPLC or GC and GC-MS. Elements were determined by inductively coupled argon plasma atomic-emission spectrometry (ICP-AES), and nitrogen was quantified using the Dumas method [159]. Total polyphenols and total free amino acids were assessed by spectrophotometry employing the Folin–Ciocalteu method [158]. Several studies included sensory parameters such as acidity, astringency, foam, taste, and mouthfeel, and physicochemical parameters like pH and total solids [162].

How local industry may be involved in science was exemplified by Bicchi et al. [157]. Based on coffee samples supplied by one of Italy's most famous coffee-roasting companies, these authors performed a thorough HPLC investigation of the caffeoyl quinic acid fraction of coffee. Extracts of green and roasted, single and blended arabica and robusta coffee beans from different geographic regions were analyzed by HPLC-UV. PCA was performed using selected caffeoyl quinic acid derivatives. Additionally, results of chemical analyses were compared with results obtained by sensory evaluation of the analyzed samples. The method employed allowed for discrimination of coffees from different geographic regions, and of arabica and robusta blends containing 20 and 80% arabica, respectively. In contrast, blends containing 40%, 50%, and 60% arabica were clearly distinguished from blends containing 20 and 80% arabica, but not from each other. Results from chemical analysis followed by PCA were in good agreement with those obtained from sensory evaluation. In conclusion, the approach chosen by Bicchi et al. gave promising results for coffee characterization with regards to origin and species [157].

A combination of chemical and sensory data was also employed by Maetzu et al. [162]. According to Maetzu et al., the sensory qualification of testing panel members is not only a question of training but also of physical and emotional factors [162]. In their investigation on espresso coffee samples, criteria for successful participation in a sensory evaluation were "good health, availability in terms of time, no aversion to coffee, and willingness to participate." Panel members selected according to these criteria were trained to assess visual parameters like foam index (i.e., the ratio of espresso foam to liquid volume) and persistence of foam of coffee brews, and to describe the coffee's taste with regards to body, acidity, bitterness, astringency, and aftertaste intensities. Sensory data of espresso samples from arabica coffee, robusta Natural blend (80% robusta, 20% arabica), and robusta Torrefacto blend (50% robusta Natural blend, 50% Robusta torrefacto, a special roast obtained by adding sugar to the coffee during roasting) were subjected to PCA together with other physicochemical parameters (e.g., pH, total solids, total lipids, caffeine, trigonelline, 5-caffeoyl quinic acid). Arabica and robusta samples were distinguished successfully based on their sensory taste parameters. The combination of organoleptic (body and foam color of the coffee) and chemical (trigonelline and 5-caffeoylquinic acid, measured by HPLC) parameters allowed for a good discrimination of the robusta Natural and Torrefacto blends.

The lipid fraction of coffee is mainly composed of triacylglycerols, free and bound fatty acids, sterols, and tocopherols [163]. A physiologically interesting substance class in the unsaponifiable lipid fraction are the diterpenes, which account for 20% of the total lipids. The main diterpenes in coffee are cafestol, 16-*O*-methylcafestol, and kahweol. Coffee diterpenes have been associated with a higher heart disease risk due to their serum cholesterol–raising effect [156,164]. Other investigations described positive health properties of coffee diterpenes, for example, a chemopreventive effect against cancer [165]. Chlorogenic acid exhibits pronounced antioxidant activity and has also been linked to the positive health effects of coffee [166]. 16-*O*-methylcafestol proved to be an efficient chemotaxonomic marker for robusta coffee, since it is contained in both the leaves and seeds of *C. robusta* but only in the leaves of *C. arabica* [150,156,167]. The German DIN 10779 standard published in 1999 for the authenticatation of coffee samples is also based on the identification of 16-*O*-methylcafestol, which was first described by Speer in 1989 [167].

Alves et al. studied in detail the differences in tocopherol content of arabica and robusta coffees [152]. Arabica beans, both green and roasted, contained significantly higher amounts of tocopherols than robusta beans. These results are in accord with results by González et al. and Kölling-Speer

et al. [160,168]. The distinction of the two source species was mainly based on β-tocopherol, which occurs in arabica in concentrations four times higher than in robusta coffee.

While Alves et al. found decreasing amounts of both α- und β-tocopherol after roasting [152], González et al. reported that roasted beans exhibited higher amounts of α- and β-tocopherol than green ones [160]. Moreover, González et al. detected considerable amounts of γ-tocopherol in their coffee samples [160]. γ-Tocopherol was not detected in any of the samples investigated by Alves et al. and only in traces in the robusta samples analyzed by Kölling-Speer et al. [152,168]. The analysis methods employed by González et al. and Kölling-Speer et al. were normal-phase HPLC and GC-MS, respectively [160,168]. The results by González et al. indicating an increase of tocopherols during the roasting process were considered to be incomprehensible and hypothesized to be caused by an inefficient extraction method and/or to be based on misidentification of the quantified compounds by Speer and Kölling-Speer and by Alves et al., respectively [152,160,163]. Although discrimination of coffee samples by means of their tocopherol profile seems to be feasible, there are still some questions to be addressed. Among them is the presence or absence of γ-tocopherol in coffee, the influence of postharvest procedures applied to the freshly collected coffee seeds, and the degeneration of tocopherols during storage.

Bertrand et al. examined the possibilities of identifying genetically enhanced arabica cultivars [159]. Arabica coffee plants are more susceptible to various diseases due to their relatively poor genetic diversity compared to robusta plants. Therefore, robusta genes of resistance have been transferred to arabica, resulting in a number of arabica lineages exhibiting higher disease resistance but also characterized by lower quality in the produced coffee. Various possibilities to distinguish genotypes of arabica with introgressed robusta genes from a traditional arabica genotype employing GC-flame ionization detector (FID) (based on determination of fatty acids), Dumas (nitrogen), ICP-AES (P, K, Ca, MG, Fe, Cu, Zn, and B), and HPLC (based on chlorogenic acids) were examined [159]. Furthermore, an attempt was made to distinguish different chemotypes due to geographic and/or environmental factors. Data were analyzed by PCA and discriminant analyses (DA). A monodirectional introgression effect was observed for 3,5-dicaffeoyl quinic acid and stearic acid, both compounds were present in lower amounts in genetically enhanced arabica cultivars, and for oleic acid, whose level was higher in the genetically modified cultivars. Performance of discriminant analysis based on fatty acid was quite satisfactory; in 79% of samples the traditional arabica cultivar Caturra was distinguished correctly from genetically enhanced cultivars. An equally good discrimination of traditional from genetically modified arabica varieties (76%) was achieved by Bertrand et al. employing near-infrared reflectance (NIR) of green coffee [169]. With only 69% correctly classified Caturra samples, discriminant analysis based on caffeoyl quinic acid derivatives was less effective [159]. The investigated elements were not useful to assign the coffee varieties. In contrast, classification of samples from different locations based on element composition was excellent (100%). The performance of DA of fatty acids (ca. 90% correct) and caffeoyl quinic acids (>80% correct) was promising. In conclusion, elements, fatty acids, and caffeoyl quinic acid derivatives proved to be valuable marker compounds within *C. arabica*. In combination, these chemical characteristics can be employed to identify genetically modified cultivars as well as the geographic origin of the investigated samples.

Casal et al. assessed trigonelline, nicotinic acid, and caffeine as chemotaxonomic markers to distinguish between arabica and robusta coffees. Though only three compounds were quantified, the authors used multivariate data-analysis techniques to analyze their HPLC results. Trigonelline and caffeine but not nicotinic acid were suited for varietal identification. None of the three compounds was correlated with the geographic origin of the analyzed samples [161].

4.5 SUMMARY/OUTLOOK

The examples from various fields of pure and applied botany, mycology, lichenology, ecology, and zoology compiled in the preceding show that chemosystematic data have interesting applications in an amazing variety of different fields. The advances in technology, especially in HPLC and the

various hyphenation techniques based on HPLC like HPLC-MS, HPLC-NMR, and HPLC-SPE-NMR, make chemosystematic studies faster and the results derived from such studies more reliable than the results from historical studies that were mostly based on PC and TLC comparisons. At the same time, the number of potential users of advanced methods decreases, especially in the field of botany, because of the high cost of the equipment. Therefore, many of the most advanced techniques have not been extensively used for pure chemosystematic studies yet. Purely chemosystematic studies in botany have also lost ground due to the advent of molecular techniques. The future of chemosytematics therefore is in our eyes most promising in the fields of marine ecology (investigation of phytoplankton), mycology (microfungi), and applied botany (food identity and food safety). In systematic botany, where chemosystematics had its origins, chemosystematic studies will still be of interest for the phenetic characterization of clades emerging from modern molecular studies. Some of these clades as, for example, in the Scrophulariceae/Plantaginaceae group, are nearly impossible to characterize morphologically, and chemosystematic data continue to help in defining and characterizing these emerging clades.

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5 Phytochemistry, Phytopharmacology, and the Biological Role of Plant Metabolites

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CONTENTS

5.1 INTRODUCTION

In all living organisms, chemical compounds are synthesized and degraded by means of many chemical reactions, each mediated by an enzyme. These processes are generally known as metabolism, which can be divided into catabolism (degradation) and anabolism (synthesis). All organisms possess similar metabolic pathways that allow them to utilize or synthesize the main chemical groups—sugars, amino acids, fatty acids, nucleotides, and their polymers: polysaccharides, proteins, lipids, and nucleic acids. These compounds are essential for survival and can be termed *primary metabolites*. Many organisms also utilize other metabolic pathways, producing compounds of no apparent utility. Such compounds are called natural products or, more often, *secondary metabolites*. This type of metabolism is especially characteristic for plant organisms, for which the diversity of compounds produced serves as a kind of immunological system. This chapter presents the systematic divisions of groups of plant metabolites as well as their roles in the natural world. For human beings, phytopharmacology is considered the most useful and important branch of research into plant metabolites. One should not, however, denigrate the role of plant metabolites, whose antifeedant, repellent, allelopathic, or other activities become important in other areas, such as ecology or agriculture.

Phytotherapy is a science-based approach to the use of medicinal plants in the treatment of diseases and is definitely the oldest area in the entire field of medicine. Plant extracts have been used for centuries as a popular method for treating various health disorders. Until the last century most medicines were derived directly from plant or animal sources [1]. In the present, there has been a renewed interest in natural products. It has been hypothesized that there are 800,000 plants, and only 15% of the 300,000 which are known, have a traditional therapeutic use, and only 1% have been verified with methods considered scientific.

5.2 PLANT METABOLITES: BIOSYNTHETIC AND CHEMICAL CLASSIFICATION; BIOLOGICAL IMPORTANCE OF PRIMARY AND SECONDARY METABOLITES

The division into primary and secondary metabolites was proposed at the begining of the twentieth century and (after certain modifications) is currently based on physiological and biochemical data [2–4]. Primary metabolites can be found in virtually each plant and are responsible for basic physiological functions—energetic, building, and reserve functions. They are indispensable for the life of each plant.

Secondary metabolites do not exhibit basic functions in the life of a plant, being only a product of specialized metabolism. Their occurrence is limited to selected smaller or larger systematic groups in the world of plants. Both the role of secondary metabolites and their interaction among organisms are currently discussed.

According to J.B. Harborne [5], secondary metabolites allow the organism to adapt to the environment in order to survive and reproduce. Adaptation to the environment consists in adjusting to the external conditions, which may bring about stress. The biochemical reaction, that is, is the production of appropriate secondary metabolites, is thus a response to stress (namely, environmental factors).

The boundary between primary and secondary metabolites is rather fuzzy—for example, many amino acids are definitely classified as secondary metabolites, whereas many steroid alcohols play an essential structural role in most organisms and must therefore be considered primary metabolites. Primary and secondary metabolites cannot be readily distinguished on the basis of precursor molecules, chemical structures, or biosynthetic origins. For example, both primary and secondary metabolites are found among diterpenes (C20) and triterpenes (C30). In the diterpene series, kaurenoic acid and abietic acid are formed by a very similar sequence of related enzymatic reactions. The former is an essential intermediate component in the synthesis of gibberellins, which are

growth hormones found in all plants, whereas the latter is a resin component largely restricted to the members of the Fabaceae and Pinaceae families.

Taking into account the inaccuracies just mentioned, the division presented in this chapter is a simplified classification of primary and secondary metabolites, based on both biosynthesis and the chemical structure of the compounds. As has been shown in the preceding examples, the biogenetic origin should not be the only factor responsible for the division of metabolites.

5.2.1 Primary Metabolites

Primary substances have the characteristics of metabolites that can be found in each plant [2,3]. The role of these substances is to play basic physiological functions. They are the source of energy and also play the role of building and reserve materials. Monosaccharides, starches, fats, chlorophylls, amino acids, proteins, and nucleic acids belong to this group.

5.2.1.1 Carbohydrates

Photosynthesis is the basic biochemical process that leads to the formation of organic matter in plants. The process is responsible for the origin of monosaccharides and their further transformation into other mono-, di-, and polysaccharides through polimerization and condensation.

Carbohydrates are natural compounds including sugars, related compounds, and their polymers, which can generally be expressed by the following formula: $C_n(H_2O)_m$.

Monosaccharides are classified according to the number of carbon atoms they contain: triose (3 carbon atoms), tetrose (4 carbon atoms), pentose (5 carbon atoms), hexose (6 carbon atoms), and heptose (7 carbon atoms), as well as by the type of carbonyl group they contain: aldose, –CHO (aldehyde) or ketose, C = O (ketone).

Disaccharides are sugars composed of two monosaccharides. There are two basic types of disaccharides: reducing and nonreducing ones. Disaccharides are formed when two monosaccharides are joined together and a molecule of water is removed. For example, milk sugar (lactose) is made from one glucose and one galactose molecule, cane sugar (sucrose) from one glucose and one fructose molecule, and maltose from two glucose molecules.

Fructo-oligosaccharides (FOS) are sugars composed of 3–10 simple sugar molecules linked by a glycosidic bond and are found in many vegetables and consist of short chains of fructose molecules. Galactooligosaccharides (GOS), which also occur naturally, consist of short chains of galactose molecules. Oligosaccharides are often found as a component of glycoproteins or glycolipids and, as such, are often used as chemical markers, often for cell recognition.

Polysaccharides are polymeric carbohydrate structures formed of repeating units (either mono- or disaccharides) and joined together by glycosidic bonds. These structures are often linear but may contain various degrees of branching. When all the monosaccharides in a polysaccharide are of the same type, the polysaccharide is called a *homopolysaccharide*, but when more than one type of monosaccharide is present, they are called *heteropolysaccharides*. Examples include storage polysaccharides such as starch and glycogen, and structural polysaccharides such as cellulose and chitin. Polysaccharides have the general formula $C_x(H_2O)_y$, where x is usually a large number between 200 and 2500. Considering that the repeating units in the polymer backbone are often six-carbon monosaccharides, the general formula can also be represented as $(C_6H_{10}O_5)_n$ where $40 \leq n \leq 3000$. Special attention should be paid to mucilages, which have the character of polymers and exhibit pharmacological activity.

5.2.1.2 Lipids

Lipids are a broad group of naturally occurring molecules and include fats, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E, and K), monoglycerides, diglycerides, phospholipids, prostanoids, and others. Biologically, lipids function mainly as a way to store energy, as structural components of cell membranes, and as important signaling molecules.

Simple lipids are derivatives of glycerol and high fatty acids, whereas complex lipids display a more complicated structure. The more important types of fatty acids that occur in lipids are the saturated ones, such as those containing C6–C24 carbon atoms, and unsaturated ones, such as fatty acids with one double bond (crotonic acid, oleic acid), two double bonds (linoleic acid), three double bonds (linolenic acid), and four double bonds (arachidonic acid).

Waxes are a kind of lipid that may contain a wide variety of long-chain alkanes, esters, polyesters, and hydroxy esters composed of long-chain primary alcohols and fatty acids. They are usually distinguished from fats by the lack of triglyceride esters of glycerin and three fatty acids such as palmitinic, stearinic and oleic. In addition to the esters, which contribute to the high melting point and hardness of carnauba wax, the epicuticular waxes of plants are mixtures of substituted long-chain aliphatic hydrocarbons, containing alkanes, fatty acids, primary and secondary alcohols, diols, ketones, and aldehydes.

5.2.1.3 Amino Acids, Peptides, and Proteins

In chemistry, an amino acid is a molecule containing both amine and carboxyl functional groups. These molecules are particularly important in biochemistry, in which the term refers to α-amino acids with the general formula $H_2NCHRCOOH$, where R is an organic substituent. Amino acids are considered to be the basic products that result from nitrogen assimilation by plants.

Amino acids are critical to life, and have a variety of roles in metabolism. One particularly important function is that they serve as the building blocks of proteins, which are linear chains of amino acids. Amino acids are also important in many other biological molecules, such as forming parts of coenzymes, as in S-adenosylmethionine, or as precursors for the biosynthesis of molecules such as heme. Due to this central role in biochemistry, amino acids are very important in nutrition.

Amino acids are the basic structural building units of proteins. They form short polymer chains called peptides or longer chains called either polypeptides or proteins. These polymers are linear and unbranched. The process of making proteins is called translation and involves the step-by-step addition of amino acids to a growing protein chain by a ribozyme that is called a ribosome. The order in which the amino acids are added is read through the genetic code from an mRNA template, which is an RNA copy of one of an organism's genes. Twenty amino acids are encoded by the standard genetic code, and these are called the proteinogenic or standard amino acids.

5.2.2 Secondary Metabolites

Secondary metabolites are the result of complicated metabolism and, as such, do not play a basic function in the life of a plant [2,4]. The function or importance of these compounds to an organism is usually of an ecological nature, as they are used to defend against predators, parasites, and diseases, to compete with other species, and to facilitate reproductive processes (coloring agents, attractive smells, etc.). Since these compounds are usually restricted to a much more limited group of organisms, they have long been of prime importance in taxonomic research. Secondary metabolites may be candidates for the development of drugs.

Most secondary metabolites that are of interest to humans fit into categories in which secondary metabolites are classified according to their biosynthetic origin. Since secondary metabolites are often created by modifying primary metabolite syntheses, or "borrowing" substrates of primary metabolite origin, the categories should not be interpreted in a general way, as saying that all molecules in a given category (for example, the steriod category) are secondary metabolites, but rather as saying that there are secondary metabolites in these categories.

Secondary metabolites can be classified on the basis of their chemical structure, their composition, the pathway through which they are biosynthesized, or their solubility in various solvents. Taking into account the biochemical pathway, secondary metabolites can be divided into the

following groups: fenolics, derived from the shickimic acid pathway; isoprenoids, derived from the mevalonate pathway; amino acid derivatives; and acetogenine derivatives.

5.2.2.1 Shickimic Acid Derivatives

Plant phenolics are biosynthesized by several different routes and thus constitute a heterogeneous group from a metabolic point of view. Two basic pathways are involved: the shikimic acid pathway and the malonic acid pathway. The shikimic acid pathway participates in the biosynthesis of most plant phenolics. The malonic acid pathway, although an important source of phenolic secondary products in fungi and bacteria, is of less significance in higher plants. Shickimic acid derivatives in plants include such secondary metabolites as phenols, phenolic acids, tannines, flavonoids, coumarins, and lignans.

5.2.2.2 Isoprenoids

Isoprenoids, sometimes called terpenoids, are a large and diverse class of naturally occurring organic chemicals similar to terpenes, derived from five-carbon isoprene units assembled and modified in thousands of ways. Most are multicyclic structures that differ from one another not only in functional groups but also in their basic carbon skeletons.

These five-carbon precursors create molecules by the synthesis of two or more units, in which the number of carbon atoms are divisible by 5, as follows:

- Monoterpenes (C10)
- Sesquiterpenes (C15)
- Diterpenes (C20)
- Sesterterpenes (C25)—rare in nature
- Triterpenes (C30)
- Tetraterpenes (C40)
- Polyterpenes (C5n)

In the group of isoprenoids there might be other molecules in which the number of carbon atoms are not divisible by 5, such as steroids and iridoids, which result from secondary reactions, such as oxidation, cyclization, or detachment of some substituents.

5.2.2.3 Amino Acid Derivatives

Amino acid derivatives include amines, which arise by decarboxylation. Amines rarely play a role as active substances in plants.

Substances that arise biogenetically from amino acids, or from their direct derivatives, are known as alkaloids. Alkaloids are naturally occurring chemical compounds containing basic nitrogen atoms. The name derives from the word *alkaline* and was used to describe any nitrogen-containing base. Alkaloids are produced by a large variety of organisms, including plants and rare bacteria, fungi, and animals. Many alkaloids are toxic to other organisms. They often have pharmacological effects.

Alkaloids are categorized into three main categories, depending on their biogenetic origin. For example, compounds that contain at least one nitrogen atom in a ring system derived from amino acids are considered true alkaloids (as opposed to alkaloids derived from phenylalanine, which are not grouped in this category). Alkaloids derived from phenylalanine are categorized as protoalkaloids, whereas others, such as steroidal alkaloids or purine alkaloids, are classified as pseudoalkaloids. Since little was known about the biosynthesis of alkaloids, they were formerly grouped under the names of the plants or animals they were isolated from.

5.2.2.4 Acetogenine (Acetylocoenzyme A) Derivatives

Polyacetylenes belong to a class of molecules containing two or more triple bonds and constitute a distinct group of relatively unstable, reactive, and bioactive natural products. They are found in

plants, fungi, microorganisms, and marine invertebrates. Polyacetylenes are common among higher plants, where they occur regularly in seven families, namely, the Apiaceae, Araliaceae, Asteraceae, Campanulaceae, Olacaceae, Pittosporaceae, and Santalaceae family, and sporadically in eight other plant families as well.

The large structural variation observed among polyacetylenes seems to indicate the involvement of many different precursors in their biosynthesis. A comparison of the polyacetylene structures with those of oleic, linoleic, crepenynic, and dehydrocrepenynic acids makes it, however, reasonable to assume that most polyacetylenes are biosynthesized with the latter acids as precursors, although there are some examples of polyacetylenes that are derived from carotenoids and other terpenoids. Many feeding experiments with ^{14}C- and ^{3}H-labeled precursors have confirmed this assumption and further speculations that polyacetylenes are built up from acetate and malonate units.

Quinones all contain the same basic chromophore, which consists of two carbonyl groups in conjugation with two carbon–carbon double bonds. Quinones can be divided into three groups of increasing molecular size: the benzoquinones, the naphthoquinones, and the anthraquinones. Many are alkylated or substituted by different lipophilic groups (isoprenyl) or hydrophilic groups (OH), free or combined with sugars. Quinones are synthesized in plants through one of four distinct pathways. Anthraquinones and naphthoquinones are mainly formed from acetate-malonate precursors or synthesized via *O*-succinylbenzoic acid. Their plant pigments range in color from yellow to black.

5.3 PHYTOPHARMACOLOGY: PHARMACOLOGICAL ACTIVITIES OF PRIMARY AND SECONDARY METABOLITES AND THERAPEUTIC APPLICATION OF PLANT MATERIALS (NATURAL DRUGS)

5.3.1 Phytotherapy of Central Nervous System (CNS) Diseases

Anxiety is one of the most common mental disorders affecting humankind. Synthetic anxiolytic substances have a lot of side effects, so very often phytotherapy plays an important role in helping to reestablish a regular sleep pattern [6]. The sedative and anxiolytic effects of valerian are used in traditional medicine to support mental relaxation and treat insomnia and anxiety. Valepotriates from the root of *Valeriana officinalis* cause a decrease in locomotor activity and possess psychostimulant, thymoleptic, and tranquilizing activity. Valeranon is a sedative and anticonvulsant, and valerinic acid inhibits metabolism of GABA. The extract stimulates the release and inhibits the uptake of GABA at nerve endings [7,8]. Likewise known for sedative and tranquilizing properties are passion flowers (*Passiflora incarnata*). The extracts decrease locomotor activity and prolong sleeping time [7,9]. Essential oil from lavender flowers acts directly on central nervous system with a sedative effect. In addition, extracts from *Melissa officinalis* or *Strobili lupuli* are known tranquilizers and have sleep-promoting activity. Extracts from *Lupuli strobulus* are used as phytomedicines to treat tension, restlessness, and sleep disorders. Very popular are combinations of *Humulus* extracts with other herbal drugs such as valerian for use as sleep-inducing drugs or with passion flower for use as sedating drugs [10]. Drugs of plant origin also provide useful support in cases of mental-health problems like depression, without the unpleasant side effects associated with prescription drugs [11]. Saint John's wort (*Hypericum perforatum*) has a stimulant effect on the CNS as a mono amine oxidase (MAO)-inhibiting agent. It also has a strong affinity for GABA receptors and inhibits serotonin uptake at synapses. Amentoflavone present in the herb has been shown to have a strong affinity for the binding site of benzodiazepines [7]. The role and the mechanisms of the different *Hypericum* constituents is still a matter of debate. *Hypericum perforatum* L. has gained widespread popularity as a natural Prozac. The therapeutic efficacy of *Hypericum* extracts is comparable to that of synthetic antidepressants, but the side effects are weaker [12]. Anxiolytic activity is characteristic of lipophilic lactones from kava kava roots, which also act as an anaesthetic and anticonvulsive. They improve sleep quality and do not depress mental and motor function. Kava kava has long been

used as a substance of abuse as it can produce feelings of euphoria and relaxation similar to alcohol. It is suggested that kavalactones activate GABA but do not alter levels of dopamine and serotonin [6,13,14].

Some herbs act as strong neuroprotective agents. *Gingko biloba* extract is widely promoted as reducing or preventing memory impairment. It exerts multiple cellular and molecular neuroprotective mechanisms: enhancing cognitive processes, improving blood circulation to the brain, attenuating apoptosis, and inhibiting membrane lipid peroxidation. Active compounds also posses anti-inflammatory activity and inhibit β-amyloid aggregation, which appear to be the main constituent of amyloid plaques in the brains of Alzheimer's disease patients [15]. Huperzine A, an alkaloid isolated from *Huperzia serrata*, a plant popular in Chinese folk medicine is also neuroprotective; it is known as a potent, selective, and well-tolerated inhibitor of AchE and prevents the generation of β-amyloids or protects against β-amyloid-induced neurotoxicity [16].

Nature provides humans with plants possessing CNS-stimulating properties. Ephedrin is attributed with stimulant effects similar to those of amphetamines. It also promotes the release and inhibits the uptake of noradrenaline. Long-term therapy may cause psychotic episodes such as paranoia or hallucinations [6]. Commonly used stimulants are plants containing methylxantines like *Coffea* sp. and *Paullinia guarana* [6].

Very important are plant adaptogens, which enable the organism to adapt to a difficult environment and increase the body's resistance to both physiological and chemical aggression. Adaptogen plants like *Panax ginseng*, *Eleutherococcus senticocus*, *Schisandra chinensis*, *Withania somnifera*, and *Rhodiola rosea* improve either the function of the CNS or the activity of the entire organism [6]. They may also be used by healthy persons for improving physical and cognitive performance and attenuating some of the disorders resulting from aging, such as loss of memory and of attention, fatigue, and general weakness [17].

5.3.2 Phytotherapy of Cardiovascular System Disorders

Nature offers us a great quantity of natural remedies for the treatment of cardiovascular system disorders. The most popular, cardiac glycosides, by inhibiting the Na/K pump cause an increase in the level of sodium in the myocytes, which then leads to a rise in the level of calcium. Higher amounts of calcium increase the force and speed of heart contractions (positive inotropic effect) and decrease the heart rate (negative chronotropic effect) [18]. Cardiac glycosides improve cardiac output and reduce distension of the heart. They are indicated for cardiac insufficiency with low output and rhythm abnormalities. The main source of this group of compounds are *Adonis vernalis*, *Convalaria maialis*, *Digitalis purpureae*, and *Digitalis lanata*. In addition, visnadin isolated from *Amni visnagae* fruits has a positive impact on the cardiovascular system. It possesses a coronary vasodilator effect and has positive inotropic, bradycardic, and spasmolytic activity so is used in preventive therapy of angina pectoris [7]. Hypotensive activity, normalization of cardiac rhythm, positive inotropic and dromotropic activity, and increases in coronary and myocardial circulation are the effects of synergism of oligomeric procyanidins and flavone *C*-glycosides (vitexin, orientin) from *Crategus monogyna* [7]. For these reasons, the extract of dried hawthorn leaves and blossoms is used to treat early stages of heart failure and arrhythmias. It acts either myocardially or peripherally to reduce vascular resistance [11].

The oxidation of LDL cholesterol and other lipoproteins is an essential step in the pathogenesis of atherosclerosis. This is why antioxidants are necessary to prevent the formation of reactive oxygen and nitrogen species, which may damage a number of essential molecules (DNA, lipids, proteins). Antioxidants may represent an important adjunct to the therapeutic choices available today to prevent the progression of atherosclerotic lesions [19]. Green tea extract contains antioxidant polyphenols important in preventing oxidation of LDL cholesterol. It significantly decreases concentrations of cholesterol and triglycerides [11]. For arterial problems such as atherosclerosis and hypertension, garlic is very useful; it has been used in traditional medicine for centuries. Sulfur

compounds are responsible for the therapeutic effect as well as garlic's flavor and odor [18]. Garlic extract inhibits cholesterol synthesis and significantly reduces its levels. The hypotensive effect of garlic and its components results from vasodilation. The extract also prevent thrombus formation and inhibits platelet aggregation—either spontaneous or induced [20]. Several studies concerning the anti-atherogenic effects of garlic showed that it is more active than synthetic drugs [20]. Procyanidins from grape seeds have an angioprotective activity. They are strong free-radical scavengers; inhibit collagenase, elastase, and hyaluronidase; and improve the functional symptoms of venous insufficiency [7]. Resveratrol—one of the main active polyphenols in red wine—is known for its strong cardioprotective effect, prevention of heart dysfunction. It is a strong antioxidant and an anti-atherosclerotic agent [21].

Very effective in cerebrovasuclar disease is extract from the leaves of *Ginkgo biloba.* Ginkgo leaves have attracted much attention as agents for improving circulation, particularly cerebral circulation, which may lead to improved brain and mental function [22,23]. Ginkgolides – isolated diterpene, are inhibitors of the platelet-activating factor (PAF), which is responsible for platelet aggregation and inflammatory reactions [7]. Biflavonoids from *Gingko* have a positive impact on vasodilation, reduce capillary vessels, and improve blood flow. In pharmacologic investigations, a significant increase in smooth muscle cGMP concentration was measured, which can be explained by a stimulation of nitric oxide (NO) release from vascular endothelial cells [24]. Biflavonoids also act as antioxidants and inhibit lipid peroxidation [11]. The extract has been described as having neuroprotective and anti-inflammatory effects as well [25].

The most effective plant used in venous disorders is *Aesculus hippocastanus* L. Aescin—a saponin glycoside—which can reduce transcapillary filtration and increase the tonus of the veins. Aesculin is said to be a venous tonic and a vascular protective agent and is used for venous insufficiency, capillary fragility, and hemorrhoidal symptoms [7,11]. Saponin glycosides from *Rusci rhizoma* (ruscine, ruscoside, ruscogenin) have a similar activity [18].

5.3.3 Phytotherapy of Respiratory System Diseases

Common minor disorders of the respiratory system can often be successfully treated with herbs. Sometimes herbs can help with some serious diseases like bronchitis or pneumonia [18].

Mucilage—presents in large quantity in *Cetraria islandica*, *Althaeae officinalis*, or *Verbascum*—forms a protective layer over the mucous membranes and acts as an antitussive and demulcent agent [11]. These herbs are very popular in pediatrics for a dry, irritated, and ticklish cough.

Essential oils from various herbs (either administered as essential oils or contained in herbal extracts) are the most important agents that directly influence secretion of more respiratory-tract fluid and mucus. Volatile oil-containing herbs like thyme, anise, eucalyptus, or fennel irritate the bronchial glands, reduce the viscosity of mucus, and act as expectorants. Moreover, they can also act as either antiseptics or spasmolytics. They are recommended for colds, coughs, and bronchitis. Essential oils can be also used as steam inhalations or chest rubs for infants and children [18]. Saponin-containing expectorant herbs evoke a reflex stimulation of respiratory secretions by activating parasympathetic sensory pathways.

Ephedrin isolated from *Ephedra sinica* is recommended for relieving bronchoconstriction in mild forms of chronic asthma. It releases a norepinephrine from symphathetic nerve endings [11]. Codeine has an anti-tussive activity, which is accompanied by a depression of the respiratory centers [7].

5.3.4 Phytotherapy of Gastrointestinal System Disorders

Plants and their derivatives can offer useful treatment alternatives for such problems as constipation, diarrhea, bowel syndrome, and liver disease. Derivates of 1,8-antraquinone (*Rheum officinale,*

Cassia angustifolia, Rhamnus frangula) are well-known stimulant laxatives. Glycosides are converted by the microbial flora of the large intestine into the active metabolites. There are two different mechanisms of action: (1) stimulation of the motility of the large intestine, resulting in accelerated colonic transit and (2) inhibition the Na-K ATPase activity, which inhibits water, sodium, and chloride resorption and stimulates the secretion of water and electrolytes into the lumen of the colon [7,11,26]. Herbal drugs with antraquinone are for short-term use in cases of occasional constipation. Long-term use of stimulant laxatives can lead to atonic colon, nausea, vomiting, diarrhea, and disorders in water equilibrium and electrolyte metabolism. Also, antraquinone glycosides are excreted in breast milk [7,26].

Phytotherapy offers not only stimulant laxatives but also bulk-producing laxatives, which, when consumed with liquid, bind water in the colon, swell to a large volume, and stimulate peristalsis and emptying of the bowel, acting as safe laxatives. The most popular are psyllium seeds, which contain a lot of mucilage that is neither absorbed nor digested in the intestines [11].

Herbal drugs can be also useful therapies in diarrhea. The polysaccharides of rice are hydrolyzed in the gastrointestinal tract where sugars are absorbed and enhance the absorption of water [18]. Blackberry, blueberry, or raspberry leaves, which contain appreciable amounts of tannins, may be considered as an astringent and also reduce intestinal inflammation [11].

Many volatile oil–containing herbs (angelica, peppermint, caraway, fennel) have spasmolytic and carminative activity. They can also stimulate gastric secretion, facilitate digestion, and promote bile flow [11]. Also, consumption of bitter herbs like gentian (*Gentiana lutea*) or centaury (*Centaureae erythrea*) stimulates the appetite and the secretion of saliva, bile, and gastric juices, so they are indicated for poor appetite and bloating [11,18]. The opposite action is characteristic of atropine and hyoscyamine, which are parasympatholytics and inhibit the muscarinic receptors, acting as competitive and reversible inhibitors of acetylcholine binding onto its receptors. As a result, these two substances decrease saliva and gastric and pancreatic secretions, as well as decreasing intestinal tonus and the frequency of peristaltic contractions [7].

Effective herbal treatments for ulcers are not numerous. The most important is glycyrrhizic acid (glycyrrhizin), a main compound from *Glycyrrhizae radix*, used to relieve gastric inflammation, mainly peptic and duodenal ulcers [18]. The most widely reported side effects of glycyrrhizin use are hypertension and edema caused by stimulation of the mineralocorticoid receptors.

Many other herbs are widely used in gastric problems. Ginger prevents symptoms of motion sickness, nausea, morning sickness during pregnancy, and vertigo. It also has carminative and spasmolytic activity [18]. Aloe gel is recommend for oral consumption as possessing benefits in various internal inflammatory conditions. It reduces the presence of yeasts and is effective in the treatment of peptic and chronic gastric ulcers [27]. Sylimarin—a mixture of flavonolignans isolated from fruits of *Sylibum marianum* (milk thistle)—has anti-hepatotoxic activity; it prevents the toxic effect of many toxins, inhibits membrane lipid peroxidation, and acts as a free-radical scavenger. In addition, it stimulates RNA-polymerase, which stimulates protein synthesis and increase tissue-regeneration capacity [7]. Milk thistle is recommended as a supportive treatment for chronic inflammatory liver conditions and cirrhosis [11].

5.3.5 Phytotherapy of Urinary System Diseases

Herbal remedies have been used for many thousands of years for resolving urinary tract infections. They can act as disinfectants, antibacterials, analgesics, and diuretics. Volatile oils, flavonoids, and saponins are typical diuretics and increase the volume of urine while keeping the level of electrolytes constant. Increasing the flow of urine also helps prevent the formation of kidney stones [11]. One of the most effective aquaretic are species from *Solidago*, which also acts as an anti-bacterial [11]. Some herbs also exhibit anti-bacterial properties, which are useful in combating infections. The most effective anti-bacterial herbs for urinary tract infection are plants from the Ericaceae

family (*Uvae ursi folium*, *Vitis ideae folium*). Arbutin, which is the main compound, after hydrolysis releases hydroquinone, which has strong anti-bacterial activity. Very useful for the prevention and treatment of infection is cranberry. The juice prevents microorganisms from adhering to the mucous membranes of the urinary tract [11]. In addition, cranberry juice increases the level of hippuric acid and therefore the acidity of the urine [18].

Herbal drugs' diuretic and anti-septic activity are only some of the known mechanisms for prevention of kidney stone formation. One of the most widely used herbal remedies is *Herniaria hirsuta*, which decreases adhesion of calcium crystals and can remove those already attached to kidney cell surfaces. *Dolichos biflorus* causes sharp decreases in phosphate and calcium precipitate formation and is popularly used in traditional medicine. Another widely used product is *Agropyron repens* extract [28].

5.3.6 Phytotherapy of Gynecological and Andrological Diseases

Over 300 plants possess compounds with estrogenic activity [11]. Plant phytoestrogens can act as mild estrogens. Isoflavonoids such as daidzein and genistein can bind to estrogen receptors and can induce similar responses to as estradiol in breast, ovarian, endometrial, prostate, vascular, and bone tissues [7,29]. Use of phytoestrogens can easily overcome hypoestrogenic symptoms like hot flashes in postmenopausal women. They play an important role in maintaining bone density and preventing or lowering the incidence of osteoporosis. Isoflavonoids and lignans reduce the risk of cardiovascular disease in postmenopausal women. They decrease the level of triglycerides and increase the metabolic score and quality of vessels [29].

In addition, some herbal drugs can regulate hormone levels without necessarily being estrogenic [18]. The rhizome and roots of *Cimicifuga racemosa* are traditionally proposed to treat premenstrual syndrome and reduce some symptoms associated with menopause [7]. Probably this kind of activity is due to substances with dopaminergic activity rather than an estrogen-like compound [30]. Methanolic and lipophilic extracts reduce the serum level of luteinizing hormone [18]. Extracts of the fruits of the chaste tree (*Vitex agnus castus*) are widely used to treat not only premenstrual symptoms (mastodynia) but also irregular cycles. It is possible that clinically important compounds possess dopaminergic activity [31]. The active flavon vitexcarpin has potent analgesic and anti-hyperprolactinemia properties, which reduce general premenstrual symptoms [32].

Phytotherapy is also as successful as synthetic drugs in treating benign prostatic hyperplasia. Extract of *Prunus africana* or *Serenoa repens* exerts an anti-androgenic effect, inhibiting 5-α-reductase and decreasing the binding between dihydrotestosterone. Phytosterols compete with androgen precursors and inhibit prostaglandin biosynthesis, penatcyclic terpenes exhibit anti-inflammatory effects, and fatty acids decrease the level of cholesterol, which can be a precursor in androgen synthesis [7,11,18]. Lignans from the roots of *Urtica dioica* inhibit the interaction between sex hormone-binding globulin and testosterone [18]. This all leads to decreases in prostate volume.

5.3.7 Phytotherapy of Rheumatic Diseases

Increasing interest in herbal drugs to treat chronic inflammatory disorders has been observed. The side effects of synthetic drugs can limit their acceptability, and plant extracts and isolated substances are very effective and are safe in therapeutic doses [18].

The secondary roots of devil's claw (*Harpagophytum procumbens*) are a traditional African herbal drug used by the natives of desert regions to treat a variety of ailments. It is known for its strong anti-inflammatory and analgesic effects. The iridoid glycosides are considered to be the predominant pharmacologically active constituents and block TNF-α activity—a cytokine involved

in systemic inflammation, which stimulates the acute phase reaction [33,34]. The extracts' main have fairly potent inhibitory effects on thromboxane biosynthesis. Because of these effects, devil's claw is used in the treatment of osteoarthritis and rheumatism; it exhibits good patient tolerability and clinical efficacy [34,35]. Boswellic acids—a mixture of penta- and tetracycline triterpene acids isolated from oleo gum resin of *Boswellia serrata*—are structurally analogous to well-known steroidal anti-inflammatory compounds in both acute and chronic inflammation models, with minimal side effects. They are also reported as an effective immunomodulatory and anti-tumor agent. Boswellic acids inhibit proinflammatory mediators in the body, specifically leukotrienes, via inhibition of 5-lipoxygenase, the key enzyme of leukotriene biosynthesis [36–38]. Organic extract of ginger (*Zingiber officinalis*) and isolated compounds decrease production of prostaglandin E_2, mostly by inhibiting the COX-2 enzyme activity [39]. Colchicine, an alkaloid that has been used for centuries in acute gouty arthritis, strongly reduces inflammation and relieves pain. It also decreases the production of uric acid. The main anti-inflammatory effect of colchicine is connected with its potent inhibitory effect on leukocyte chemotaxis. Decreasing the expression of adhesion molecules on neutrophil membranes stops the migration and modulation of cytokine production [40]. *Verbena officinalis* is widely used in folk medicine as an antirheumatic because of its strong anti-inflammatory and analgesic activity; it is taken orally but also externally as ointments or compresses [41]. Willow-bark extract is also recommend for treating rheumatic disorders. Extracts demonstrate analgesic and anti-inflammatory effects as well. Responsible for this activity—especially for the inhibition of proinflammatory cytokines—are salicilin and related glycosides [42]. Capsaicin, a phenolic derivative from *Capsicum annuum* L., when applied locally is effective in intractable pain [11].

5.3.8 Phytotherapy in Dermatology

Herbal treatment of dermatitis involves removing irritating and allergenic agents, so the application of astringent compounds is very important [11]. Leaves of *Hamamelis virginiana* or *Quercus cortex* contain tannins, which reduce the permeability and secretions of inflamed tissue and form a protective layer on skin [11].

Some linear furanocoumarins such as psoralen, 5-methoxypsoralen (5-MOP), and 8-methoxypsoralen (8-MOP) are photosensitizing agents when activated by near-UV light (PUVA therapy). They intercalate into DNA and form light-induced adducts with pyrimidine bases [43]. The DNA damage induced by the treatment inhibits cell division and leads to beneficial effects in therapy of psoriaris. PUVA therapy is used for repigmentation in patients with vitiligo [44]. Natural products such as bergamot oil (containing 5-MOP) are photosensitizers in sun lotion because they increase the number of melanocytes and melanin production [7]. Hydroquinone and its derivatives inhibit melanin synthesis; therefore, it is used for the treatment of melanin hyperpigmentation and in skin-bleaching cosmetics [7].

Extracts from a number of *Aloe* species have been reported as having therapeutic dermatologic properties useful in healing wounds, sunburn, and thermal injury (also frostbite). In addition, it acts as an anti-inflammatory and anti-fungal agent, stimulates fibroblast activity and collagen proliferation, and increases levels of hyaluronic acid. It is known that polysaccharides act as nonspecific immunostimulants [45,46].

The therapeutic value of *Symphytum officinale* roots is attributed to their allantoin content—a soothing and anti-irritant agent that promotes cell proliferation and wound healing and is a known skin protector [11]. Extract from flowers of marigold (*Calendula officinalis*) acts as an anti-inflammatory agent and is used in wound healing. This activity is due to the lipophilic triterpene alcohols. In addition, its essential oil exerts anti-bacterial and anti-fungal effects [18]. *Arnica montana* extract has been shown to have antimicrobial, anti-edema, and anti-inflammatory properties. It is recommended for treatment of pain and to improve wound healing [11].

5.3.9 Phytotherapy of Cancer

Most cancer drugs that are used clinically are either natural products or owe their origin to a natural source [18]. The most significant discovery was taxol, a mitotic spindle poison. It promotes the assembly of tubulin dimers into microtubules, which it stabilizes by inhibiting their depolymerization, and can be used to treat ovarian and breast cancer and non-small cell lung cancer [7,18]. Anti-mitotic activity is attributed to vinbalstine and vincristine, which are alkaloids isolated from *Vinca minor.* They inhibit mitosis by binding to tubulin and cause an accumulation of cells in the metaphase. They are used in therapy for different kinds of cancer, mostly in complex combination chemotherapy [7,18].

Another agent, camptothecin from *Camptotheca acuminata*, is promising in treatment of a number of different cancer types like gastric, liver, head, and bladder cancers [18]. Camptothecin itself is not used clinically due to its cytotoxicity, but its derivatives are highly effective for the treatment of cancer [47]. The dietary intake of glucosinolates (from broccoli, cabbage, and brussel sprouts) might have a protective effect against colon cancer [7].

It is known that consuming high amounts of phytoestrogens lowers rates of several cancers including breast, prostate, and colon cancer. It may be due to their role in lowering circulating levels of unconjugated sex hormones. Dietary supplementation with soy isoflavonoids was shown to increase the levels of sex hormone-binding globulin in postmenopausal women, lowering the serum levels of free-sex hormones [29]. Genistein modulates genes involved in homeostatic control, induces apoptosis, and inhibits the growth of cancer cells [48]. Also, isoflavones and lignans are able to inhibit enzymes involved in the synthesis of steroid hormones [49].

5.3.10 Immunomodulatory Activities of Plant Materials

Several different substances from above-ground parts of *Echinacea purpurea* have been identified that may contribute to its immunomodulatory effects, such as polysaccharides, caffeic acid derivatives, alkamides, and melanins. It is very possible that a combination of these and other unknown agents contributes to the overall therapeutic activity of *Echinacea* products. All compounds are a strong stimulator of various immune cells such as macrophages, other monocytes, and natural killer (NK) cells and can be use in colds, respiratory and urinary tract infections, and poorly healing wounds. The *E. pallida* root (fresh or dried) has been approved for use in the treatment of influenza-like infections [7,50,51]. Immunomodulatory activity was confirmed also for acemannan, a polysaccharide from *Aloe vera* gel. It was reported to activate macrophages, enhance cytokine release, and increase lymphocyte responses. Polysaccharides may exhibit anti-tumor and anti-viral activities through enhanced immune response and immunomodulation [52,53].

5.3.11 Toxicity

Although most plant possess beneficial activity, some of them contain very strong active substances that may cause dangerous side effects. The phallotoxins and amatoxins—cyclopeptides present in toadstool species like *Amanita phalloides* and *A. verna*—inhibit RNA polymerase II, which blocks synthesis of proteins and ultimately leads to cell death. They are the most potent liver toxin known, causing necrosis of the liver and, to a lesser degree, the kidney [18]. Strong hepatotoxicity is also characteristic of pyrrolizidine alkaloids present in *Tussilago farfara*, *Symphytum officinale*, or *Borago officinale*. It seems to be due to the biotransformation of unsaturated alkaloids into unstable, toxic metabolites, probably pyrrolic derivatives, by cytochrome P-450 [54]. According to experimental studies, the amount of pyrrolizidine alkaloids present in herbal remedies is sufficient to be potentially carcinogenic in exposed individuals [55]. Thujone, a major component of the essential oil of *Artemisia absinthium* and present in leaves of *Salvia officinalis* as well, is neuro- and nefrotoxic and hallucinogenic in large doses. It also causes convulsions. The *Cicuta*

genus includes the most violently toxic poisonous plants known. The main compound is cicutoxin, an unsaturated aliphatic alcohol that is most concentrated in the roots. The toxin acts directly on the central nervous system, causing extreme and violent nausea, vomiting, convulsions, and death from respiratory failure [18]. Tropane alkaloids like atropine, hyoscyamine, or scopolamine lead to anti-cholinergic poisoning. Symptoms usually occur 30–60 minutes after ingestion and may continue for 24–48 hours because tropane alkaloids delay gastric emptying and absorption. They act as competitive antagonists to acetylcholine at both peripheral and central muscarinic receptors. Toxic doses of atropine or hyoscyamine cause agitation, disorientation, hallucinations, and mental confusion [7].

Cardiac glycosides due to their cumulative effect can easily give rise to toxic symptoms. Any dysrhythmia characterized by both increased automaticity and depressed conduction is suggestive of cardiac glycoside toxicity. A few minutes after administration of a toxic dose of aconite, an alkaloid present in *Aconitum napellus*, gastrointestinal signs appear together with tingling and numbness. After severe vomiting, death occurs from asphyxia. The aconitine toxin is absorbed easily through the skin. Aconitine is a potent neurotoxin [18].

Strychnine isolated from the seeds of the *Strychnos nux vomica* tree is also extremely toxic. This alkaloid produces some of the most dramatic and painful symptoms of any known toxic reaction. Several minutes after exposure, muscles begin to contract and spasm, typically starting with the head and neck and spreading diffusely. The convulsions are nearly continuous and can increase in intensity with the slightest stimulus. Death occurs from asphyxiation caused by paralysis of the neural pathways that control breathing, or from sheer exhaustion due to the severity of the muscle convulsions. A patient will die within several hours of exposure [18].

5.4 OTHER ACTIVITIES OF NATURAL METABOLITES

As was presented at the begining of this chapter, secondary metabolites are the measures an organism uses to adapt to the environment in order to survive and reproduce [5,56,57]. Thus, adaptation to the environment consists in adaptation to external factors evoking stress. A biochemical reaction is a response to stress (namely, environmental factors).

We have taken into account plants and their metabolites, as plants seem to be richer in their biochemical diversity than animals. It is true that secondary matabolism is a phenomenon that is also observed in animals. Nevertheless, more than four-fifths of all known natural compounds are derived from plants, which results from the fact that plants are immobile and cannot react in the ways animals can.

Environmental factors influencing plants can be divided into five groups:

1. Climatic factors: temperature, humidity, light intensity, day length
2. Edaphic (soil) conditions: chemical composition of soils including salinity, heavy metal salts toxic to the plant, deficiency of mineral components
3. Unnatural contaminants that arise as a result of human activity: environmental pollutants in air, soil, and water
4. Animals: the major plant enemies, specifically herbivores (Many different defense adaptations are known. Symbiosis—in the case of pollinating animals—has also been observed.)
5. Competition with other plants including microorganisms and higher plants

The classification of interactions between plants and other organisms can be based on the preceding division of environmental factors (mainly points 4 and 5). Thus we talk about the so-called phytochemical ecology. Interactions between organisms can be divided into following types: plant–plant, plant–animal, and animal–animal (the last issue is not discussed here, since it does not relate to the thematic scope of the book).

5.4.1 Plant–Plant Interactions

The term *allelopathy* derives from two Greek words: *allelo* "interact (mutually)" and *pathos* "experience, feeling, sensitivity, suffering." The term relates mainly to the excretion of chemical compounds into the soil. These compounds inhibit the growth of other organisms in the surroundings (mainly plants and microorganisms), and can induce or impede germination, as well as the growth and development of other plant species living in their company or occupying the same place immediately after them.

It is a kind of antagonistic interaction between populations that consists of a population A producing a substance that is harmful for a competing population B. Numerous experiments carried out in the 1930s and later indicated that particular species growing in the vicinity can exert a stimulatory or inhibitory influence on plants of other species, or on microorganisms and fungi.

In the case of allelopathy, there are two types of interactions:

a. gas or liquid secretions produced by the above-ground plant parts, such as leaves, flowers, and seeds, acting directly on the neighboring plants, bacteria, or fungi
b. root secretions that penetrate the soil and act indirectly

The phenomena described within the allelopathy framework occur among all plant classes, including microorganisms. Employing an division of "allelopatically active" organisms into microorganisms and higher plants, the substances secreted by the respective groups have been ordered as follows:

1. *Kolins (interaction between higher plants).* These are chemical compounds—volatile elements (ethylene, essential oils, alcohols, and others), liquids (solutions of glycosides, alkaloids, etc.), and solids (tannins)—that are produced by higher plants and disturb the growth and development of other higher plants. *Juglans nigra* is an example of such an interaction. Its influence on other plant species was observed long ago, as the majority of plants that were growing within several meters of it died. *Juglans regia* seems to reveal similar features. This phenomenon results from the existence of a well-known chemical compound, juglone, that becomes active after being present in the soil for a certain period and can be deposited to 2 m in depth.

   The action of allelopathic compounds in a plant organism is to inhibit germination, limit protein synthesis, and decrease the supply of assimilates, which causes plugging of the capillaries in the plant. These substances also have other activities, such as the retardation of the formation of root nodules in plants from the Fabeaceae family.
2. *Antibiotics (interactions between microorganisms).* Antibiotics, secreted mostly by fungi, are a widely known example of allelopathic interactions. The biological task of antibiotics is to inhibit the growth of bacteria and other fungi. Antibiotics play a bacteriostatic or germicidal role, disturbing the synthesis of proteins, nucleic acids, and bacteria cell-membrane components.
3. *Marasmines (influence of microorganisms on higher plants).* These are products of metabolism that are produced by microorganisms; they injure higher plants, most often causing perturbation in the water balance and rapid wilting of plants. They can also modify the growth of plants, plugging plant vessels, or decompose plants' defense substances. Plant growth hormones, mucilages, enzymes, citric acid, oxalic acid, and so on are examples of marasmines.
4. *Phytocides (the influence of higher plants on microorganisms).* Derived from Greek *phyton* ("plant") and Latin *caedo* ("killed"), phytocides refers to volatile substances produced by living plants in leaves, flowers, seeds, and roots. Phytocides have germicidal, fungitoxic, and protozoan toxic properties or inhibit microorganisms' growth. Such examples as

allicine (garlic), alkaloids (quinnie or emetine), and others are produced by higher plants, cause the inhibition of plant growth, and disturb microorganisms' metabolic processes.

Numerous papers emphasize the role of phytocides in the purification of air from bacteria and also their function in air ionization. The phytocide activity of greenery—its vital hygienic property—is conditioned by the species of a plant, its age, growth stage, and the intensity of photosynthesis and also depends on climatic and soil environmental conditions. Plants that have been damaged (by prunning, fendage, haymaking, etc.) tend to secrete higher amounts of phytocides.

Another option concerning both the division and the naming of the interactions between higher and lower plants emphasizes the existence of compounds labeled as phytoalexines and phytotoxines (pathoxines). According to this option, the interactions between a higher plant and a microorganism reveal the attack of a microorganism on a higher plant and thus contribute to the disease of such a plant (e.g., decay, canker, maculation, etc).

The classification of the higher plants' resistance to diseases is as follows.

1. Preinfectious compounds
   a. Prohibitines: metabolites limiting or fully inhibiting in vitro growth of microorganisms, including the following examples:
      i. Terpenoids or phenolics accumulated in ligneous tissues of coniferous and deciduous trees
      ii. Hydroxystilbenes (e.g., pinosylvin) that confer immunity to fungal infections; found in the Pinaceae family
      iii. Catechine and protocatechuic acid, found in fungus-resistant onion varieties
      iv. Numerous groups of flavonoids that exhibit anti-fungal activity in sufficient strength to inhibit fungal growth (e.g., the erivatives of flavone present in leaves of citrus fruits)
   b. Inhibitines: metabolites whose content has to increase after infection in order for them to carry out their toxic activity (for example, the inhibition of the growth of the fungus *Phytophthora infestans* by scopoline and chlorogenic acid in potato tubers)
2. Postinfectious compounds
   a. Postinhibitines: metabolites that arise from existing inactive compounds by their hydrolysis or oxidation
      i. Cyanogenic glycosides (occurring, for example, in plants from the Fabaceae family) that are nontoxic for pathogens but, under the influence of an enzyme known as β-glucosidase, hydrolyze to a hydrocyanic acid
      ii. Glucosinolates found in the Brassicaeae family (for example, synigrine in the *Brassica* genus, which transforms to volatile allil isothiocyanide and is the resistance factor against the fungal pathogen Phyllactinia)
   b. Phytoalexins: metabolites synthesized de novo after the invasion via gene derepression or the activation of the enzymatic system (A widely known example of a phytoalexin is pisatin, the derivative of pterocarpane, which is a compound synthesized in the *Pisum sativum* pod when infected by conidiospores of fungus causing *Monilinia fructicola*. Phaseolin is another pisatin derivative produced by the bean. The production of phytoalexins is also charcteristic for plants belonging to following families: *Convolvulaceae* [ipomeamaron] and *Orchidaceae* [orchinol in orchid bulbs].)

5.4.2 Plant–Animal Interactions

Attractants, or attracting substances, are physical factors or chemical compounds (natural or artificial) used to increase attractiveness. Attractants also have a practical application in animal farming,

plant cultivation, and plant protection. Moreover, the flavoring and coloring factors play an important role as attractants in the biochemistry of plant pollination.

For insects, the role of attractants is played by metabolites from almost all chemical groups, with the prevalence of toxic substances or repellents; it has also been stated that in most plant–insect systems more than one nutritive attractant exists. Examples of such compounds include the previously mentioned glucosinolates, cyanogenic glycosides, bitter cucurbitacins, or sparteine (a toxic alkaloid), as well as many other compounds.

Repellents, also called discouraging agents (Latin *repellere*, "discard, discourage"), are living organisms, chemical compounds, or other factors that repel undesirable species in a given place. Their use in horticultural practice is considered to be an organic (ecological) method of protection. A repellent activity manifests in an unpleasant smell or in caustic, corrosive substances contained in a given repellent that may attack the legs or the respiratory system.

It often happens that the metabolites of certain plants are repellents for the vermin of other plants. For example, tomato plants protect cabbages against *Pieris brassicae*, and onions protects carrots against *Psila rosae*. Examples of other practical application of repellents include volatile compounds present in *Ledum palustrae* that repel moths and dimethylphthalane, which is an effective repellent against *Aedes aegypti* and *Anopheles quadrimaculatus.*

Antifeedants are nutritive deterrents, that is, substances that partially or completely hamper insects from preying on plants but are usually nontoxic for them or toxic to a small degree. They paralyze taste organs and cause the preying to cease, which results in the starvation of insects that are still close to the food source. The decrease of fertility, or the prolonging of the larval stadium, and the like have also been observed as the effects of antifeedants. The most representative antifidants belong to various groups of chemical substances and are obtained from natural sources. Their production on industrial scale is difficult and unprofitable. Therefore, the use of synthetic substances similar to natural derivatives that might contribute to limiting the population of certain insects is still being investigated.

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6 Sample Preparation of Plant Material

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CONTENTS

6.1 PRELIMINARY PROCESSING OF PLANT MATERIAL

6.1.1 Collecting and Harvesting

The collection of wild plants is still very important in the preparation of plant materials [1]. Workers who collect medicinal plants must demonstrate knowledge of plant species. Unfortunately, the intensive collection of wild plants often leads to excessive destruction of some species. Increased industrialization and environmental pollution also reduce the wild plant habitat. As a result, sometimes the transfer of wild species into cultivation is needed.

In comparison with the harvesting of wild plants, cultivation has many advantages. It allows the collection of large quantities of raw materials from a relatively small area. Moreover, it means that the plant material has similar morphological characteristics and similar content of active components. Another advantage of the method is the ability to mechanize the work of harvesting plants and the possibility of placing the plantations in convenient locations (e.g., near the pharmaceutical industry). Cultivation provides numerous benefits, providing, as a result of a single origin of the seed and the conditions of plantation, the raw material more balanced, both in terms of development and chemical composition. Cultivation provides numerous benefits such as: single origin of the seed and the conditions of plantation, raw material is more balanced, both in terms of development and chemical composition. Moreover it aspires to the creation of such cultures of medicinal plants, which interferes with the genetic structure of selected plants, providing new opportunities. Special development of breeding and its role dates from the time when the phytochemical investigation have shown enormous variability in the chemical composition of herbal raw materials and related ambiguous therapeutic activity. It also turned out that breeding procedures may lead to an increase content of pharmacologically active compounds in raw material, which are genetically determined.

The quantitative composition of a plant's active components depends on the time of harvest (the season and even the time of day), as the content of these substances is not constant throughout the year. Plant samples are collected from the aerial parts (e.g., herbs, leaves, fruits, seeds, stems, and stem bark), the trunk bark, and roots [2]. Usually, leaves are collected just as the flowers are beginning to open, flowers are collected just before they are fully expanded, and the underground elements are gathered as the aerial parts die down. Moreover, aerial parts of plants (leaves, flowers, fruits) should not be collected when they are covered with dew or rain. Plants that are discolored or have been attacked by insects or slugs also should not be collected.

6.1.2 Drying

Drying is a very important process for the quality of plant material [1]. The temperature, air movement, and thickness of the raw material have an important role here. The plants are usually dried in the air in the shade or in different types of heated driers. Better control is achieved in the laboratory using drying cabinets or lyophilizers (freeze-dryers), although biomass has to be dried quickly to avoid degradation of the components by air temperature or by microbes. The drying process can also be carried out using a vacuum drying method or by infrared rays.

Lyophilization is a kind of drying achieved by freezing the wet substance, then causing the ice to sublime directly to vapor by exposing it to a low partial pressure of water vapor. Because lyophilization is the most complex and expensive method of drying, its application is usually restricted to delicate, heat-sensitive materials.

The drying temperature influences the content of metabolites in the plant material [1]. Natural plant materials that contain volatile compounds—for example, essential oils (families Lamiaceae, Asteraceae), esters (valepotriates from Radix Valerianae), vitamins (mainly vitamin C), enzymes, and hormones—are particularly sensitive to higher drying temperatures. In these cases a drying temperature of 30–35°C is required. Sometimes gradual drying can be appied, for example, first at a temperature of 20–30°C and then at 80–100°C (for cardiac glycosides).

In order to retain sensitive compounds (enzymes, hormones), often the lyophilization drying process by sublimation of water in a vacuum at a low temperature should be applied. Drying conditions for medicinal plants are usually given in pharmacopoeias (e.g., in the European Pharmacopoeia).

6.1.3 Storage

Appropriate storage of plant material is essential to prevent loss of biologically active compounds. Particular components belong to various chemical groups, and they have different volatility and physicochemical properties such as susceptibility to oxidation, hydrolysis, polymerization, and

isomerization [1]. For example, the volatile compounds found in essential oil include carbohydrates, alcohols, terpenoid aldehydes, ketones or esters. Unsaturated compounds (like polyphenols) easily undergo oxidation, glycosides and esters may undergo a hydrolysis process, and catechin tannins undergo a polymerization process.

A basic condition for proper storage is the placing of plant material in a dry room at a low temperature. Air humidity has a significant influence on the storage of plant material. In most cases, in spite of adequate storage conditions, reductions in the amount of active compounds in plant material can be observed. Plant materials containing cardiac glycosides lose more than 50% of their activity within 10 days, when the air humidity is 80%. Alkaloid and glycoside plant materials usually lose a few percent of their active ingredients in 5 or 10 years, and tannin plant materials lose 20% in 2 years.

Another problem is protection against pests. On a small scale chloroform vapor is applied as an insecticide, but on a large scale tetrachloride carbon, ethyl bromide, or dichloroethane is used. However, these compounds are toxic, and they must be removed from plant material by ventilation. The water content of the plant also effects changes in plant materials. The water content is important for the action of hydrolyzing enzymes and for the growth of microorganisms.

Alkaloid plant material are fairly stable, except for tropane alkaloids, which change during the drying process (the racemization process of hyoscyamine to atropine). Plant materials containing cardiac glycosides are unstable, and the changes (hydrolysis) depend on the water present in the plant. Fermentation and oxidation processes often occur in plant material containing anthraglycosides. Plant materials containing tannins are susceptible to oxidation, so they do not have to be stored in the powder form. Vegetable material containing tannin must be stored in the form of raw material, and should be conducted in the form of powder just before use. Flavonoids in raw plant materials are sensitive to the light, oxidation, and hydrolysis reactions, and oils in plant materials decay under the influence of higher temperatures.

The time and conditions of storage are usually specified in pharmacopoeial rules. Also, humidity limits for individual plant materials are determined by pharmacopoeias.

6.1.4 Homogenization

After the drying process plant materials have to be fragmented into small particles using either a blender or a mill [2]. Plant material should be milled twice, first using a coarse mill and then a fine mill to generate a fine powder. The grinding process is important since effective extraction depends on the size of the biomass particles. Large particles will be poorly extracted, whereas small particles have a higher surface area and will thus be extracted more efficiently.

6.2 LIQUID–SOLID EXTRACTION METHODS

6.2.1 Traditional Methods: Maceration, Infusion, Decoction, Percolation, and Boiling under Reflux

Maceration, infusion, and decoction are usually performed using water to obtain extracts from dry plant materials. Maceration is carried out by immersing plant material in a particular volume of water at room temperature and leaving it in a closed vessel for 30 min, often while mixing it. Next, it should be strained using a filter and supplemented the filtrate to determined volume. Often the exhaustive maceration process, which aims at the total extraction of the raw material, is carried out.

In order to prepare an infusion, the plant material should be placed in suitable dish, and then it has to be immersed in hot water and boiled in a water bath for 15 min. Next, the solution has to be left for 15 min and stirred from time to time. After that, the contents of the dish should be filtrated and supplemented to determined volume.

Decoctions are performed as follows: The plant material should be flooded with water at room temperature, mixed, and placed in a water bath. Next, the mixture should be warmed to the temperature 90°C for 30 min. The percolation process is carried out as follows: First, the plant material should be moistened with solvent and placed in a closed vessel for 2 h. Next, the wet material is placed in a percolator for 24 h in order to macerate. After that, typical percolation by regulation of solvent flow should be done. The percolation is complete when the liquid flowing out of the percolator is almost colorless and flavorless.

Boiling under reflux is another method of isolating components from plant extracts. This process is based on differences in the volatility of particular elements of the mixture. Distillation transforms the substance into the vapor state, and then the condensate is collected in a receptacle after flowing through the radiator.

As cited in the literature, the maceration and percolation processes are most commonly used, while infusion, decoction, and distillation under reflux are used rarely. Examples of applications of these methods of extraction are given in Table 6.1 [3–85].

6.2.2 Soxhlet Extraction

Soxhlet extraction was named after Baron von Soxhlet, who introduced this method in the nineteenth century [86]. It was the most common leaching technique used for extracting active substances from plant material until novel extraction methods were developed. Actually, it is the main reference against which the performance of other extraction techniques is compared [87].

In this method, the solid sample is placed in a thimble holder that is gradually filled with the leaching liquid phase (Figure 6.1). Refluxing extraction solvent condenses into the thimble and

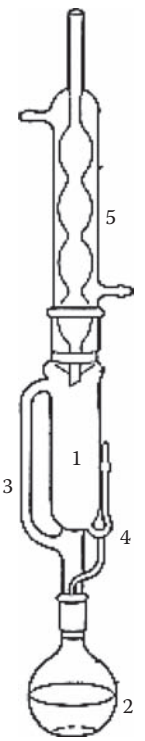

FIGURE 6.1 Experimental Soxhlet extraction apparatus. 1, porous thimble; 2, solvent in flask; 3, pipe; 4, siphon; 5, condenser. (Adapted from Kou, D. and Mitra, S., *Sample Preparation Techniques in Analytical Chemistry*, John Wiley & Sons, Hoboken, NJ, 139–183, 2003.)

TABLE 6.1
Application of Maceration, Percolation, Infusion, Decoction, and Boiling under Reflux in Phytochemistry Analysis

Substance Type, Plant Species, and Analyzed Part	Solvent Type	Time and Temperature	Ref.
Maceration			
Flavonoids, *Cyperus capitatus*, underground parts	Chloroform, methanol	—	[3]
Biflavonoids, *Plagiomnium undulatum*, gametophytes	80% methanol	—	[4]
Flavonoids, *Tanacetum parthenium*, aerial parts	Ethyl acetate	24 h at room temperature	[5]
Isoflavonoids, flavone glycosides, *Iris carthaliniae*, rhizome	90% ethanol	At room temperature	[6]
Acetophenones, chalcone, chromone, flavonoids, *Pancratium martimum*, flowering bulbs	Ethanol	72 h	[7]
Flavonoids, chalcones, *Combretum albopunctatum*, aerial parts	Dichloromethane	At room temperature	[8]
Flavans, flavanones, chalcones, dihydrochalcones, pyrrolophenanthridone, alkaloids, *Crinum bulbispermum*, bulbs	Ethanol	96 h	[9]
Flavonols, *Pterogyne nitens*, fruits	Ethanol	At room temperature	[10]
Flavonoids, *Macaranga conifera*, leaves	Methanol	—	[11]
Flavanones, flavones, *Muntingia calabura*, leaves	Methanol	2 days at room temperature	[12]
Flavonoids, *Macaranga triloba*, leaves	Methanol	—	[13]
Flavones, *Primula veris*, flowers	Methanol	36 h, 40°C	[14]
Saikosaponins, *Bupleurum gibraltaricum*, *Bupleurum spinosum*, roots	Ethanol	—	[15]
Saponins, *Ilex paraguariensis*, leaves, twigs	Ethanol–water (6:4)	—	[16]
Triterpenoid saponins, *Lafoensia glyptocarpa*, leaves	Hexane, methanol	Exhaustive extraction at room temperature	[17]
Triterpene saponins, *Chenopodium quinoa*, flowers, fruits, seed coats, and seeds	Methanol	Exhaustive extraction	[18]
Triterpenoidal saponin glycosides, *Glinus lotoides*, herb	Methanol	At room temperature	[19]

(*Continued*)

TABLE 6.1 (CONTINUED)
Application of Maceration, Percolation, Infusion, Decoction, and Boiling under Reflux in Phytochemistry Analysis

Substance Type, Plant Species, and Analyzed Part	Solvent Type	Time and Temperature	Ref.
Saponins, *Silene jenisseensis*, root	95% ethanol	—	[20,21]
Saponins, *Taverniera aegyptiaca*, stem and root bark	Methanol	At room temperature	[22]
Triterpenoid saponins, *Pteleopsis suberosa*, stem bark	Chloroform, then chloroform–methanol (9:1), then methanol	48 h, exhaustive extraction	[23]
Steroidal saponins, *Tribulus alatus*, aerial parts	Methanol	Exhaustive extraction	[24]
Steroidal saponins, *Tribulus pentandrus*, aerial parts	80% ethanol	Exhaustive extraction at room temperature	[25]
Alkaloids, *Narcissus pseudonarcissus*, bulbs	Methanol	At room temperature	[26,27]
Pyrrolizidine alkaloids, *Anchusa strigosa*, flowers, leaves, and roots	Chloroform, methanol	48 h, exhaustive extraction	[28]
Caffeine, *Ilex paraguariensis*, leaves	Hexane, methanol	10 days	[29]
Diterpenes, α-spinasterol, chlorogenic acid, isochlorogenic acid, isoquercitrin, geranylgeraniol derivatives, *Baccharis ochracea*, aerial parts	Dichloromethane–methanol (9:1), then ethanol–water (2:3)	—	[30]
Pentacyclic triterpenes, γ-lactone, serratolide, tormentic acid, *Myrianthus serratus*, trunk wood	Methanol	3 days at room temperature	[31]
Diterpenes, *Pinus taeda*, wood	Ethanol	—	[32]
Pentacyclic triterpenes, *Combretum imberbe*, leaves, *Terminalia stuhlmannii*, stem bark	Dichloromethane	At room temperature	[33]
Phenylpropanoid glucosides, *Chrozophora oblique*, aerial parts	Methanol	At room temperature	[34]
Benzofuran glycosides, *Psoralea plicata*, seeds	Methanol	At room temperature	[35]
Tetralones, *Zygogynum* species, bark, leaves	Hexane, ethyl acetate, methanol	Sequential extraction at room temperature	[36]
Antraquinones, *Vismia laurentii*, fruits	Hexane, ethyl acetate, methanol	Exhaustive extraction	[37]
Megastigmane glycosides, *Trifolium alexandrinum*, seeds	Hexane	—	[38]

TABLE 6.1 (CONTINUED)
Application of Maceration, Percolation, Infusion, Decoction, and Boiling under Reflux in Phytochemistry Analysis

Substance Type, Plant Species, and Analyzed Part	Solvent Type	Time and Temperature	Ref.
Phenolic cinnamate dimer, *Psoralea plicata*, herb	75% methanol	Exhaustive extraction	[39]
Thyme oils, *Thymus eriocalyx*, *Thymus X-porlock*, aerial parts	Water	4 h	[40]
Quercetagetin 7-methyl ether glycosides, *Paepalanthus vellozioides*, *Paepalanthus latipes*, leaves and scapes	Chloroform, 80% methanol	At room temperature	[41]
Acylated quercetagetin glycosides, *Tagetes maxima*, leaves	Methanol	At room temperature	[42]
Clerodane diterpenoids, *Premna tomentosa*, leaves	Methanol	At room temperature	[43]
Xanthones, *Vismia laurentii*, roots	Methanol–dichloromethane (1:1)	Exhaustive extraction at room temperature	[44]
Cyclotide, *Viola odorata*, aerial parts	Dichloromethane	24 h	[45]
Sesquiterpene coumarins, *Ferula szowitsiana*, roots	Acetone	Exhaustive extraction at room temperature	[46]
Diterpenes, triterpenes, sesterterpenes, *Salvia palaestina*, aerial parts	Hexane, acetone	Exhaustive extraction at room temperature	[47]
Stilbene, glucoside dimmers, *Picea abies*, bark	70% aqueous acetone	Exhaustive extraction at room temperature	[48]
Rotenone, *Derris elliptica*, *Derris malaccensis*, stem and root	Chloroform	3 days at room temperature	[49]
Percolation			
Flavonoids, *Sideritis* species	Hexane; ethanol–water (7:3)	At room temperature	[50]
Isoflavanones, *Erythrina sacleuxii*, stem bark	Dichloromethane	—	[51]
Flavanone glycosides, *Alhagi pseudalhagi*, the whole plant	Methanol	25°C	[52]
Flavonoids, *Erythrina burttii*, stem bark	Acetone	—	[53]
Flavonoids *Erythrina abyssinica*, stem *bark*	Ethyl acetate	—	[54]
Flavanones, *Erythrina burttii*, stem bark	Chloroform	—	[55]
Norditerpenoid alkaloids, *Delphinium formosum*, aerial parts	Ethanol	—	[56]

(*Continued*)

TABLE 6.1 (CONTINUED)
Application of Maceration, Percolation, Infusion, Decoction, and Boiling under Reflux in Phytochemistry Analysis

Substance Type, Plant Species, and Analyzed Part	Solvent Type	Time and Temperature	Ref.
Diterpenoid alkaloids, *Delphinium crispulum*, aerial parts	Methanol	Exhaustive extraction	[57]
Dihydroagarofuran alkaloid triterpenes, *Maytenus heterophylla*, *Maytenus arbutifolia*, aerial parts and stem bark	95% ethanol	At room temperature	[58]
Indole and carbazole alkaloids, *Glycosmis montana*, twigs and leaves	Methanol	3 weeks at room temperature	[59]
Carbazole alkaloids, *Murraya koenigii*, roots	Chloroform	—	[60]
Piperidine alkaloids, *Piper methysticum*, aerial parts	Ethyl acetate	3 days at room temperature	[61]
Quinoline alkaloids, *Psychotria glomerulata*, aerial parts	Methanol	—	[62]
Indole alkaloids, *Nauclea officinalis*, stem bark	Methanol	3 weeks at room temperature	[63]
Diterpenoid alkaloids, *Consolida glandulosa*, aerial parts	70% ethanol	At room temperature	[64]
Norditerpenoid alkaloids, *Delphinium* species, aerial parts	80% ethanol	Exhaustive extraction at room temperature	[65]
Labdane diterpenoids, *Turreanthus africanus*, seeds	Methylene chloride	At room temperature	[66]
Labdane diterpenoids, *Aframomum zambesiacum*, seeds	Petroleum ether, chloroform, methanol	At room temperature	[67]
Triterpenoids, *Gentiana neurotheca*, leaves	Methanol	—	[68]
Triterpenes, *Rhus taitensis*, leaves	Petrol, dichloromethane, methanol	At room temperature	[69]
Steroidal saponin, *Allium tuberosum*, seeds	Petroleum ether	—	[70]
Rotenoid derivatives, *Derris trifoliate*, seeds	Methanol	—	[71]
Norditerpene furan glycosides, *Tinospora cordifolia*, stems	Methanol	—	[72]
Clerodanes, *Cleidion spiciflorum*, roots	Hexane	—	[73]
Diarylheptanoids, *Myrica arborea*, stem and root bark	Methanol–dichloromethane (1:1)	—	[74]
Lignans, *Echinops giganteus*, roots	Dichloromethane–methanol (1:1)	At room temperature	[75]

TABLE 6.1 (CONTINUED)
Application of Maceration, Percolation, Infusion, Decoction, and Boiling under Reflux in Phytochemistry Analysis

Substance Type, Plant Species, and Analyzed Part	Solvent Type	Time and Temperature	Ref.
Infusion			
Flavonoid glycoside, *Maytenus aquifolium*, leaves	Water	—	[76]
Glucosinolates, *Brassica oleracea*, seeds	Water	5 min	[77]
Phenolic acids, flavonoids, *Mentha spicata*, *Tilia europea*, *Urtica dioica*, *Hypericum perforatum*, aerial parts	Water	30 min	[78]
Flavonoids, *Sorocea bomplandii*, leaves	Water	10 min	[79]
Decoction			
Geniposide, glycosides, iridoid glycosides, *Gardenia jasminoides*, fruit	Water	5 min and then 15 min	[80]
Proanthocyanidins, *Uncaria tomentosa*, bark	Water	40 min	[81]
Boiling under reflux			
Alkaloids, *Peganum nigellastrum*, aerial parts	Ethanol	3 times for 2 h	[82]
Acylated anthocyanin, *Begonia*, flowers	Methanol–ethanol (1:1)	—	[83]
Acylated flavonol glycosides, *Eugenia jambolana*, leaves	Hot 70% methanol	—	[84]
Triterpene saponins, *Filicium decipiens*, stem bark	Methanol	3 hours	[85]

extracts the soluble substances. When the liquid reaches a preset level, a syphon aspirates the whole contents of the cavity and unloads it back into the distillation flask, carrying the extracted analytes in the bulk liquid. The process is repeated until the analyte has been removed from the solid sample and concentrated in the flask. Since the extractant is recirculated through the sample, the process has a continous capacity [88,89].

Soxhlet extractions are usually slow (6 h or more), but the system operates unattended. The most common extractors use hundreds of milliliters of solvent, but small extractors are available for milligram-size samples. This type of solid–liquid extraction mainly depends on the nature of the matrix and the size of particles, as internal diffusion may be a limiting factor during the process. Method development consists of finding a volatile solvent in which the analyte is highly soluble and the matrix is poorly soluble. Different solvents (methanol, isopropanol, ethanol, hexane, hydrocarbons, and even water) can be used as extractants. Addition of cosolvents to increase the polarity of the liquid phase is possible [89,90].

The most crucial advantages of Soxhlet extraction are the facts that fresh, hot extraction solvent is always provided to the sample, thus causing maximum analyte solubility, and that no filtration

is required after finishing the process. It is a very simple, inexpensive method that can extract more sample mass than most of the latest methods. Wide industrial applications, better reproducibility, and good efficiency are the advantages of Soxhlet extraction over new extraction methods [87,90].

The drawbacks involved in the use of this conventional technique are the long time of extraction and the inability to stir the mixture, which would help accelerate the process. The potential of thermal decomposition of thermolabile analytes cannot be ignored, as the process usually remains at the boiling point of the extractant for a long time. The extract volume is relatively large, so a solvent-evaporation step is usually needed to concentrate the analytes prior to extract cleanup and analysis. Moreover, solvents used for the extraction are expensive and cause environmental problems [86,88,90]. Soxhlet extraction is a generally well-established and popular technique, and series of scientists have tried to improve it. These modifications aimed at applying Soxhlet extraction to a special type of sample (e.g., thermolabile ones) and developing new designs of basic units. In 1994, automated Soxhlet extraction (Soxtec, commercially) was approved as a standard method. In order to reduce the time and amount of solvent and to improve the efficiency of auxiliary extraction features such as a vacuum pump, a membrane-separation unit, or a source of ultrasound and microwaves, supercritical fluids can be incorporated into the conventional Soxhlet process [86,87,90,91].

Extraction in a Soxhlet apparatus (15 h, 2-g sample, methanol as extractant) was the most efficient method of isolating flavonoids (rutin and isoquercitrin) and phenolic acids (protocatechuic, *p*-hydroxybenzoic, vanillic, ferulic) from *Sambucus nigra* L. inflorescence [92,93]. In case of extraction of furanocoumarins from the *Archangelica officinalis* fruits and *Pastinaca sativa* fruits (15 h with petroleum ether as extractant, then 15 h with methanol, 10-g sample) this method gave a high yield comparable to the accelerated solvent extraction (ASE) method [94,30]. This method also gives a good extraction yield of nicotine from tobacco, sideroxylonas from eucalyptus foliage, and parthenolide from *Tanacetum parthenium* [95–97].

6.2.3 Ultrasound Assisted Extraction (USAE)

USAE relies on application of ultrasonic waves with a minimum frequency of 16 kHz. This type of extraction uses ultrasonic vibration to exert a mechanical effect, allowing greater penetration of solvent into the sample matrix, increasing the contact surface area between the solid and the liquid phase [88,98,99]. Usually, ultrasonic effects are much more intense in heterogeneous than in homogeneous chemical systems. The use of higher temperatures (which improves solubility and diffusivity) and higher pressure (which improves penetration and transport) can increase the efficiency of the extraction process [88,100,101].

Acoustic impedance depends on the characteristics of the solvent used (e.g., polarity, viscosity, vapor pressure). Increasing the polarity of the system (including solvents, analytes, and matrix) increases the efficiency of the extraction, which is why a cosolvent is sometimes added. The use of USAE is advisable for thermolabile analytes. Extraction is independent of the matrix and relatively fast, and the equipment is inexpensive [87,98,102,103].

Drawbacks of USAE are the inability to change the solvent during the process and the mandatory filtration step after extraction. The efficiency of USAE is not as high as that of some other techniques. Also, it has been reported that ultrasonic waves may cause decomposition of some compounds [86,87].

This type of extraction has been applied to biological matrices such as plant materials. In addition, other processes can also be aided by ultrasonic energy, for example, liquid–liquid extraction (LLE) [88,104]. USAE has been used to extract bioactive compounds from plants. Isoquercitrin from *Sambucus nigra* L. inflorescence is extracted well by the USAE method at 60°C. For rutin extracted from *Polygonum aviculare*, ultrasound- and microwave-assisted methods give significantly higher yields than long-duration exhaustive Soxhlet extraction [92]. USAE was found to be fast and reliable and gave better results than the mix-stirring technique for the extraction of isoflavone glucosides from soybeans. Large amounts of isoflavones are extracted with ethanol (50%) in 10 min at 60°C,

and quantitative recoveries are obtained after 20 min [103]. The ultrasonic extraction technique was shown to be very efficient in the extraction of ferulic acid from *Polygonum aviculare* [93] and of chlorogenic acid from *Eucommia ulmodies* Oliv. [98]. Sonification appears to have great potential as a method for the extraction of antioxidants (e.g., carnosic acid) from *Rosmarinus officinalis* [101]. In the case of more hydrophilic furanocoumarins—xanthotoxin and isopimpinellin from *Pastinaca sativa* fruits and xanthotoxin from *Archangelica officinalis* fruits—USAE is the most effective of all extraction methods examined [94,105]. In the case of tropane alkaloids (atropine and scopolamine) from *Datura innoxia*, the highest extraction yield was obtained by use of USAE with aqueous acetic acid or aqueous tartaric acid at 60°C [106].

Ultrasonication was a critical pretreatment to obtain high yields of oils from almonds, apricots, and rice bran. The yield of oil extracted from soybeans also increased significantly using ultrasound. Extraction rates of carvone and limonene by USAE with hexane were 1.3–2 times more rapid than for conventional extraction, depending on the temperature. Furthermore, the yield and purity of carvone obtained by USAE were better than those achieved by a conventional method. USAE of ginseng saponins occurred about three times faster than traditional Soxhlet extraction [90,102]. In the extraction of steroids and terpenoids from three *Chresta* spp. (*C. exsucca*, *C. scapigera*, and *C. sphaerocephala*) the use of ultrasound decreased significantly the total time of treatment; in addition, this extraction method was more effective than maceration [99].

Ultrasound-assisted extraction was considered as an efficient method for extracting bioactive compounds from *Salvia officinalis* and *Hibiscus tiliaceus L.* flowers [90], hemicellulose from buckwheat hulls [107], reserpine from *Rauwolfia serpentina*, and pyrethrin from *Chrysantheum cineraria* [108].

6.2.4 Microwave-Assisted Solvent Extraction (MASE)

MASE was first carried out in 1986 by Ganzler et al. to extract fats from food and pesticides from soil. The use of microwaves as a modern extraction method has been reported by Paré and López-Ávila using focused and multimodal microwaves (microwave-assisted process [MAP] and MASE) [87,90].

Microwaves are electromagnetic radiations with a frequency from 0.3 to 300 GHz that can penetrate biomaterials and interact with polar molecules such as water in the biomaterials to create heat. Consequently, microwaves can heat a whole material to penetration depth simultaneously. Unlike conventional heating based on conduction and convection, microwave heating is connected with the dielectric constant. Generally, absorption of the energy increases with the dielectric constant of the molecule, resulting in more effective heating. In microwave radiation, electromagnetic energy is transformed into heat through ionic conduction [86,87,95].

MASE can be performed in a closed or open system. The closed MASE system is used for a microwave-absorbing extraction solvent (high-dielectric constant). The sample and the solvent are placed in a closed non-microwave-absorbing vessel. The closed MASE system is generally used for extraction under drastic conditions; microwaves heat the extractant to a temperature higher than its boiling point. The pressure in the vessel (up to 5 atm) essentially depends on the volume and the boiling point of the solvents. The open-vessel system, also known as the atmospheric pressure microwave or focused microwave system, can be operated at a maximum temperature determined by the boiling point of the solvents at atmospheric pressure. The vessel, made of glass or quartz, is usually connected to a condenser to reflux the volatile analytes and solvent [86,90,109,110].

The efficiency of MASE is influenced by factors such as the solvent, matrix effects and water content, temperature, and extraction time [86]. The accurate choice of solvent is the key to successful extraction. Solvents with high-dielectric constant (ethanol, methanol, water) are sufficiently polar to be heated by microwave energy. Solvents with low-dielectric constants (hexane, toluene) are not potential solvents for MASE. The extracting selectivity and the capacity of the extractant

to absorb microwaves can be modulated by using mixtures of solvents (e.g., hexane–acetone) or of non-polar solvents with a small amount of water (e.g., 10%). The sample, which contains water or other high-dielectric components, absorbs the microwave radiation and releases the heated analytes into the surrounding liquid, which is selected for good analyte solubility. The efficiency of MASE is significantly influenced by plant particle size and, consequently, the contact surface between the plant matrix and the solvent. The particle sizes of the extracted materials are usually in the range of 0, 1–2 mm [89,90].

Generally, recovery increases with the increases in temperature and then levels off after a certain point. High temperatures may cause degradation of thermolabile compounds and decomposition of the matrix. Generally, pressure is not a critical parameter in MASE. Many microwave extractions can reach maximum recovery in 10–20 min. Longer periods of extraction may lead to the decomposition of thermolabile analytes [86].

MASE has been considered as a potential alternative to traditional solid–liquid extraction methods. In a typical application 2–20 g of a sample is loaded into an extraction vessel with a certain amount of a selected solvent. The extraction takes 10–20 min, and then the vessels are cooled for about 10–20 min. Finally, the extract is filtered, concentrated, and analyzed [86,90].

The major advantages of microwave extraction over conventional methods are its high efficiency, shorter time and lower solvent usage, high precision, and repeatability. In this method a large number of samples can be processed simultaneously. At the same time, MASE has several disadvantages: For example, the efficiency of microwaves is poor when either the analytes or the solvents are nonpolar or when they are volatile; cooling and filtration are necessary after the extraction; thermal degradation and chemical reactions are possible; and the equipment is expensive [86,111,112].

MASE using the open system (80% methanol in water) gives good yield of furanocoumarin from *Archangelica officinalis* fruits. In the case of xanthotoxin, isopimpinelin, and phellopterin, the yield is comparable to or even higher than the yield obtained using exhaustive Soxhlet extraction [94]. In the case of *Sambucus nigra* L. inflorescence, the highest yields of chlorogenic and *p*-coumaric acids and high yields of rutin and isoquercitrin were noted in MASE using the open system (80% methanol in water). Closed-system MASE gives the highest extraction yield of protocatechuic, *p*-hydroxybenzoic, and gallic acids and high yields of isoquercitrin from *Polygonum aviculare* [92,93]. A new focused MASE method has been developed for leaching geniposidic and chlorogenic acids from *Eucommia ulmodies* Oliv. [110].

MASE (80% methanol) could dramatically reduce the extraction time of ginseng saponins from 12 h using conventional extraction techniques to a few seconds [90]. It took only 30 s to extract cocaine from coca leaves with the assistance of microwave energy quantitatively similar to those obtained in several hours by conventional solid–liquid extraction [113]. Microwave extraction of puerarin from the herb *Radix puerariae* could be completed within 1 min. This method was efficient in recovering approximately 95% of the total capsaicinoid fraction from capsicum fruit in 15 min compared with 2 h for the reflux and 24 h for the shaken flask methods [90].

MASE using ethanol and 95% ethanol in water as extractants was the most efficient method for the extraction of tanshinones from the roots of *Salvia miltiorrhiza bunge*. The results showed that the percentage of tanshinones was extracted by MASE and in fact higher than that of conventional extraction methods. MASE takes only 2 min, whereas extraction at room temperature, heat reflux extraction, ultrasonic extraction, and Soxhlet extraction take 24 h, 45 min, 75 min, and 90 min, respectively [114].

For the extraction of polyphenols and caffeine from green tea leaves, a 4-min microwave radiation obtained a higher extraction yield than an extraction at room temperature for 20 h, USAE for 90 min, and heat reflux extraction for 45 min, respectively [115]. A short MASE (4–15 min) also achieved a higher extraction yield than conventional extraction methods (10–25 h) for the isolation of artemisinin from *Artemisia annua L*, glycyrrhizic acid from licorice root, and ginsenosides from ginseng root [90].

6.2.5 Accelerated Solvent Extraction (ASE)

ASE is also known as pressurized liquid extraction (PLE). It uses common organic solvents at high temperature (50–200°C) and pressure (between 10 and 1500–2000 psi) to extract soluble analytes from solid samples. ASE was introduced in 1995 as a consequence of many years of research into supercritical fluid extraction (SFE). SFE is matrix dependent and often requires the addition of organic modifiers. ASE was developed to overcome these limitations [86]. Analyte recovery is enhanced and accelerated by the higher temperatures, and solvent volume can by reduced because of the high-solute capacity of the heated solvents. Also, pressure allows the extraction cell to be filled faster and helps to force liquid into the solid matrix. Elevated temperatures enhance the solvent's diffusivity, resulting in increased extraction kinetics [89,90].

A typical schematic diagram of an ASE system is given in Figures 6.2 and 6.3. It consist of a solvent tank, solvent pump, extraction cell, heating oven, collection vial, and nitrogen tank. The ASE system is fully automated. The plant material (5–20 g) is mixed with neutral glass and loaded into the extraction cell, and then the solvent is pumped in. The application of a neutral material (e.g., sand, silica, glass) playing the role of dispersion agent is recommended to reduce the volume of solvent used for the extraction. The extractant is delivered from one or more bottles. Then the extraction cell (made of stainless steel) is heated to the desired temperature and pressure. The heat-up takes 5–9 min. When the pressure reaches 200 psi above the preset value, the static valve opens to release the excess pressure and then closes again. After heating, the extraction can be conducted dynamically, statically, or as a combination of both. In the dynamic mode, the extraction solvent flows

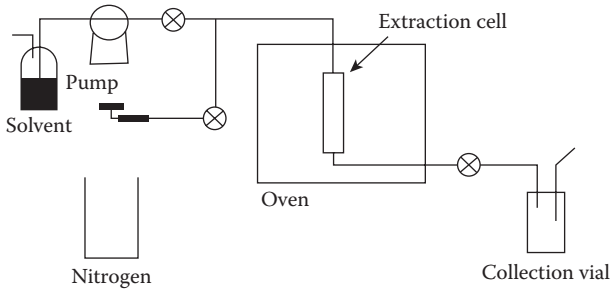

FIGURE 6.2 Schematic diagram of accelerated solvent extraction (ASE) system. (Adapted from Kou, D. and Mitra, S., *Sample Preparation Techniques in Analytical Chemistry*, John Wiley & Sons, Hoboken, NJ, 139–183, 2003.)

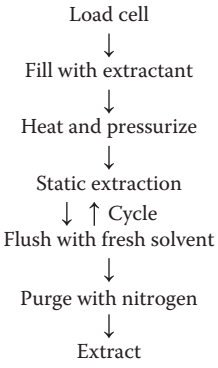

FIGURE 6.3 Schematic diagram of accelerated solvent extraction (ASE) processing. (Adapted from Kou, D. and Mitra, S., *Sample Preparation Techniques in Analytical Chemistry*, John Wiley & Sons, Hoboken, NJ, 139–183, 2003.)

through the system, whereas there is no solvent flow in the static mode. After extraction, the extract is flushed into collection vials with fresh extractant [86,89].

The general criteria for the solvent selection are high solubility of the analytes and low solubility of the sample matrix. Solvents used in conventional extraction methods can readily be applied in ASE. However, solvents that are not efficient in Soxhlet extraction may yield high recovery under ASE conditions. Pressurized hot water or subcritical water can also be used in an ASE apparatus. Then the method is usually called pressurized hot water extraction or subcritical water extraction.

ASE has many advantages. The small sample size can reduce the solvent volume. This method is fast, fully automated, and easy to use. Filtration takes place during the process, so additional filtration is not needed. However, ASE conditions may lead to degradation of thermolabile compounds. Despite the high-equipment price, the cost per sample can be relatively low [86,90,116].

ASE is usually used for the extraction from plant materials of substances that are stable at high temperatures, such as taxoids (10-deacetylbaccatin III, paclitaxel and cephalomanine) from *Taxus baccata* var. *aurea* [117]. The method (petroleum ether and then methanol as extractants, 10 min, 100°C, 60 bar) gives, in most cases, higher yields of furanocoumarins from *Pastinaca sativa* and *Archangelica officinalis* fruits than exhaustive extraction in Soxhlet apparatus [94,105]. In the case of more hydrophobic furanocoumarins (bergapten, imperatorin, and phellopterin), it is the most effective of all the extraction methods examined (Soxhlet extraction, MASE, USAE). PLE (methanol, 100°C, 60 bar) gives the best results in comparison to other methods in the extraction of rutin and isoquercitrin from *Polygonum aviculare* [92].

ASE was developed for the rapid extraction of cocaine and benzoylecgonine from coca leaves using methanol as a solvent. The optimal pressure, temperature, extraction time, and particle size were found to be 20 MPa, 80°C, 10 min, and 90–150 μm. ASE gives similar results to Soxhlet (in terms of recovery, repeatability, and selectivity) in the extraction of steroids from the leaves of *Iochroma gesnerioides*. Extraction time and solvent consumption were dramatically reduced with ASE [90]. Higher extraction efficiency compared to USAE and good method precision were achieved for the isolation of berberine from *Captidis rhizoma* and of aristolochic acids from *Radix aristolochiae* using ASE with methanol as an extractant [116].

In the extraction of the curcuminoids (phenylpropane derivates) from *Curcuma xanthorrhiza* rhizome, three extraction cycles with methanol at 80°C were carried out. The first cycle already extracted 98% of the curcuminoids. Exhaustive ASE was the most efficient method for the extraction of hypericin from *Hypericum perforatum* herbs. After removal of undesirable lipophilic constituents by one extraction cycle with dichloromethane, hypericin was extracted by three cycles with methanol (at 50–100°C). The yield of total diantrons was higher using ASE than using the traditional methods [118]. ASE could dramatically reduce the time needed to extract escin (triterpene saponins) from *Aesculus hippocastanum* seed, from 7 h using traditional procedures to 22 min. In the isolation of thymol from *Thymus vulgaris*, ASE was carried out with one 5-min cycle of extraction with hexane at 50°C followed by a 5-min cycle with dichloromethane at the same temperature. Quantitative determination of thymol gave comparable results to 2-h steam distillation. For the isolation of silybin from *Silybum marianum* fruit, a 10-min ASE obtained a higher extraction yield than an extraction in a Soxhlet apparatus taking several hours. The solvent consumption for ASE was five times lower than for Soxhlet method [118].

6.2.6 Supercritical Fluid Extraction (SFE)

Supercritical fluids were discovered by Baron Cagniard de la Tour in 1822. The use of supercritical fluids for analytical purposes started with capillary supercritical fluid chromatography (SFC), which was introduced by Novotny et al in 1981. Analytical-scale SFE became commercially available in the mid-1980s [86].

A supercritical state is achieved when the temperature and the pressure of a substance are raised over its critical value. The supercritical fluid has characteristics of both gases and liquids [90]. The

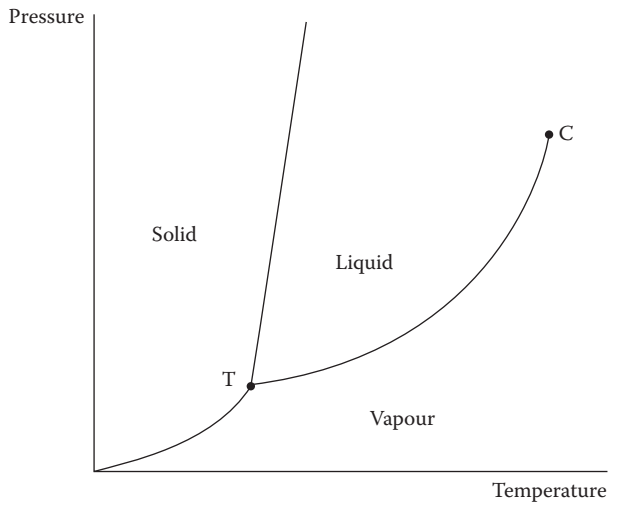

FIGURE 6.4 Phase diagram of a pure substance. T, triple point, C, critical point. (Self, R., *Extraction of Organic Analytes from Foods*, The Royal Society of Chemistry, Cambridge, UK, 2005. With permission.)

TABLE 6.2
Critical Parameters of Select Substances

Substance	Critical Pressure (atm)	Critical Temperature (°C)
CO_2	72.9	31.3
N_2O	72.5	36.5
H_2O	227	374
NH_3	112.5	132.5
SF_6	37.1	45.5
n-C_4H_{10}	37.5	152
n-C_5H_{12}	33.3	197
CHF_3	46.9	25.9
CCl_2F_2	40.7	112

Source: Adapted from Kou, D. and Mitra, S., *Sample Preparation Techniques in Analytical Chemistry,* John Wiley & Sons, Hoboken, NJ, 139–183, 2003.

physical state of a pure substance shows a phase diagram that defines regions corresponding to the solid, liquid, and gaseous states (Figure 6.4). Beyond the critical point, in the supercritical region, a gas does not liquefy under increasing pressure [86,89,119]. Compared with liquid solvents, supercritical fluids have several advantages. The dissolving power of a supercritical fluid depends on its density, which can be adjusted by changing the pressure and temperature. Substances in a supercritical state have a higher diffusion coefficient and lower viscosity and surface tension than a liquid solvent, leading to more favorable mass transfer [90].

Fluids that can be used for SFE include CO_2, NH_3, N_2O, pentane, freons, organic solvents, and others (Table 6.2). CO_2 is nonflammable, nontoxic, chemically inert, noncorrosive, and available in a highly pure form at a reasonable cost. CO_2 is easily removed from the analyte collected and causes no disposal problems [89]. Therefore, supercritical CO_2 is the preferred solvent for many supercritical extractions. The CO_2 must be free of water, hydrocarbons, and halocarbons [119]. Being nonpolar and without permanent dipole moment, supercritical CO_2 is a good solvent for the extraction of nonpolar and moderately polar compounds. Polar compounds are poorly soluble in carbon

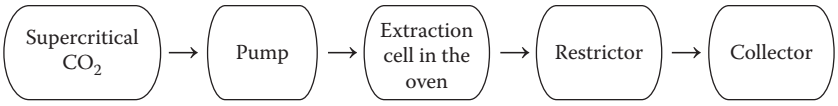

FIGURE 6.5 Schematic diagram of supercritical fluid extraction (SFE) system. (Adapted from Venkat, E., and Kothandaraman, S., *Natural Product Isolation*, Humana Press, Totowa, NJ, 91–111, 1998.)

dioxide and hence not extractable. This problem has been overcome either by adding small quantities (1–10%) of polar organic cosolvent, to increase the polarity of the extractant, or by reducing the polarity of the analytes to be leached by complex or ion-pair formation or by esterification. Both alternatives have been used separately or in combination [86,87]. Common modifiers are methanol, ethanol, acetonitrile, acetone, water, hexane, diethyl ether, and dichloromethane. Methanol is the most often used because it is an effective polar cosolvent and is up to 20% miscible with CO_2 [90].

A schematic diagram of an SFE system is shown in Figure 6.5. The essential parts include a source of CO_2, a high-pressure pump, an extraction cell, a heating oven, a flow restrictor, and an analyte-collection device (normally a vessel). The temperature is separately controlled for the pump head, the extraction chamber, the restrictor, and the collection device. The pump used in SFE must generate high pressure (from 3500 to 1000 psi), deliver reproducible volumes, and supply a constant flow rate (at least 2 mL/min) [86,89].

During SFE, raw plant material is loaded into an extraction vessel, which is equipped with temperature controllers and pressure valves at both the inlet and outlet to maintain the desired extraction conditions. The extraction cell is made of stainless polyether ether ketone (PEEK) or any other suitable material. The extraction cell is placed usually in an oven that can heat up to 200° C.

Preparation of plant materials is critical factor for SFE. Fresh materials are frequently used when SFE is applied to plant material. Wet matrices may require water removal for good recovery and reproducibility of analyte extraction. The high-moisture content can cause mechanical difficulties such as restrictor clogging due to ice formation. Although water is only about 0.3% soluble in supercritical CO_2, highly water-soluble solutes would prefer to partition into the aqueous phase, resulting in low efficiency of SFE. Adding sodium sulfate and silica gel and mixing them thoroughly with plant materials has been reported to remove the moisture. Plant particle size is also important for the SFE process. While the process is controlled by internal diffusion, large particles may cause a long-extraction time. However, fine powder can speed up the extraction but may also entail difficulty in maintaining a proper flow rate. Therefore, some rigid inert materials such as glass beads are often packed with the fine plant powder to maintain the desired permeability of the particle bed [86,90,119]. The pressure of the supercritical fluid is controlled by the restrictor. The fluid and the dissolved compounds are transported to separators. In on-line collections, the analytical instrument is usually coupled to the extraction equipment. The analyte can be trapped at the head of the column in supercritical fluid chromatography (SFC), gas chromatography (GC), or high performance liquid chromatography (HPLC) and then analyzed. Off-line collection is simpler, and the collected sample can be analyzed by several methods.

SFE has many advantages. This method is relatively fast and uses a minimum amount of solvent (5–10 mL). Filtration and evaporation steps are not required, and SFE can be directly coupled with a chromatographic method for simultaneously extracting and quantifying highly volatile compounds. However, SFE is matrix dependent and has limited applicability. In this method, sample size is small (<10 g), and the instrument is rather expensive [86,90,119].

SFE is a potential alternative to conventional extraction methods using organic solvents for extracting biologically active components from plants. It has been used to extract plant materials, especially lipids, essential oils, flavors, and fat-soluble vitamins. SFE can prevent the oxidation of lipids. In SFE of hazelnut oil, the unstable polyunsaturated fatty acids were more protected against oxidation than the *n*-hexane-extracted oil [90]. The content of essential oil obtained from *Matricaria chamomilla* flowers by supercritical CO_2 extraction was 4.4 times higher than that

achieved by hydrodistillation taking 4 h [120]. The yield of supercritical CO_2 extraction of essential oil from juniper wood was 14.7% (w/w), while hydrodistillation gave a yield of 11% (w/w). The mean percentage yields of cedarwood oil for supercritical CO_2 extraction and steam distillation were 4.4 and 1.3%. Moderate supercritical CO_2 conditions could give an efficient extraction of *Foeniculum vulgare* volatile oil, enabling about 94% of the oil to be extracted within 150 min. Compared with hydrodistillation, SFE led to higher concentrations of light oxygenated compounds in the oil extracted from *Egytian marjoram* leaves [90]. Also, SFE of fitosterols and triterpenes from roots of *Taraxacum officinale* Web achieves better results than traditional methods of extraction [121]. The extraction with supercritical CO_2 of the sesquiterpene lactone parthenolide from feverfew plant was compared with steam distillation and solvent extraction. While SFE extracted the less volatile lactones and parthenolide, the steam distillation extracted the volatile terpenes. SFE resulted in incomplete extraction compared to solvent extraction. The addition of 4% methanol resulted in complete extraction but with coextraction of chlorophyll and other poorly volatilized components [119].

Comparison of different extraction methods for the extraction of oleoresin from dried onion showed that the yield after supercritical CO_2 extraction was 22 times higher than that after steam distillation, but it was only 7% of the yield achieved by extraction using a polar solvent at 25°C. Supercritical CO_2 extraction with methanol (3–7%) was proven to be a very efficient and fast method to recover more than 98% of colchicines and colchicoside, and 97% of 3-demethylcolchicine, from seeds of *Colchicum autumnale* [90].

SFE gives good results in the extraction of lipids from *Eucalyptus globulus* wood [122], of naringin (a glycosylated flavonoid) from *Citrus paradise* (9.5 MPa, 58.6°C), of epicatechin from sweet Thai tamarind seed coat [90], of taxol from Pacific yew tree, and of cyclosporine from the fungus *Beavaria nivea* [119].

Table 6.3 gives information about the advantages and disadvantages of extraction methods, and Table 6.4 compares the extraction methods applied for the isolation of metabolites from plant material.

6.3 SAMPLE PURIFICATION AND CONCENTRATION

6.3.1 Liquid–Liquid Extraction (LLE)

LLE is a solvent extraction technique applied to liquids (liquid samples or samples in solution). This method is useful for separating analytes from interferences by partioning the sample between two immiscible liquids or phases. In practice, one phase is usually aqueous, while the other phase is an organic solvent (Figure 6.6). An extraction can be accomplished if the analyte has favorable solubility in the organic solvent [123]. Ideally, the separations are quantitative, but a perfect separation is never possible. Since the extraction is an equilibrium process with limited efficiency, meaningful quantities of the analyte can remain in both phases.

The theory of LLE is based on the Nernst distribution law. This law states that any species will distribute between two immiscible solvents so that the ratio of the concentration remains constant:

$$K_D = \frac{C_o}{C_{aq}}, \tag{6.1}$$

where K_D is the distribution constant, C_o the concentration of the analyte in the organic phase, and C_{aq} the concentration of the analyte in the aqueous phase. The fraction of the total amount of analyte extracted in the organic phase is given by the extracted fraction E:

$$E = \frac{C_o V_o}{C_o V_o + C_{aq} V_{aq}} = \frac{K_D}{1 + K_D V}, \tag{6.2}$$

TABLE 6.3
Advantages and Disadvantages of Extraction Methods

Method	Advantages	Disadvantages
Soxhlet extraction	Not matrix dependent Unattended operation Rugged benchmark method Filtration not required Inexpensive equipment	Slow extraction Large amount of solvent used Mandatory evaporation of extract
Ultrasound assisted extraction (USAE)	Not matrix dependent Fast extraction Large amount of sample Relatively inexpensive equipment	Large amount of solvent used Extraction efficiency not as high Labor intensive Mandatory evaporation of extract Filtration required
Microwave-assisted solvent extraction (MASE)	Fast extraction High sample throughput Large amount of sample Small amount of solvent	Polar solvents needed Cleanup mandatory Filtration required Degradation and chemical reaction possible Expensive equipment
Accelerated solvent extraction (ASE)	Fast extraction Large amount of sample Small amount of solvent Filtration not required Automated Easy to use	Cleanup necessary Expensive equipment
Supercritical fluid extraction (SFE)	Fast extraction Minimal solvent use Carbon dioxide is nontoxic and environmentally friendly Controlled selectivity Evaporation not needed Filtration not required	Matrix dependent Small sample size Limited applicability Expensive equipment

Source: Adapted from Kou, D. and Mitra, S., *Sample Preparation Techniques in Analytical Chemistry*, John Wiley & Sons, Hoboken, NJ, 139–183, 2003.

where V_o is the volume of the organic phase, V_{aq} is the volume of the aqueous phase, and V is the phase ratio V_o/V_{aq}. For one-step extractions K must be large (e.g., > 10). In most cases, quantitative recoveries require two or more extractions [89].

The LLE process can be accomplished by shaking the aqueous and organic phases together to mix them in a separatory funnel. Commonly, separatory funnels are globe-shaped, pear-shaped, or cylindrical (Figure 6.7). They may be shaken manually or mechanically. The flow from the bottom is controlled by a stopcock. With the stopcock closed, both phases are added to the separatory funnel. Following mixing, the layers are allowed to separate. When the layers are completely separated, the lower layer should be drawn off through the stopcock, and the top layer should be removed through the top of the separatory funnel. The location of the layers depends on the the relative densities of the two immiscible solvents (usually aqueous and organic solvents). Solvents that are denser than water will form the lower layer, while solvents that are less dense than water will form the upper layer. During an extraction process, the layers should be saved until the desired analyte is isolated [123].

A variant of the extraction process is continuous LLE, where fresh solvent is continually recycled through the aqueous sample (Figure 6.8). This procedure is typically used when the extraction

TABLE 6.4
Comparison of Different Methods for Selected Compounds

Phytochemicals	Plants	Extraction Method	Solvent	Extraction Time (min)	Yield (g/kg matrix)
Naringin	*Citrus paradisi*	Soxhlet	Ethanol + water	480	15.2
		SFE	CO_2 + ethanol	45	14.4
Saponins	Ginseng	MASE	80% methanol in water	0.5	53.1
Tocols	*Amaranthus caudatus*	USAE 25°C	Methanol	60	0.0637
		SFE	CO_2	15	0.129
β-Sitosterol/ α-tocopherol/ γ-tocopherol	Okra	Soxhlet	*n*-Hexane	—	2.01/0.127/0.380
	seed	Soxhlet	Ethanol	—	2.68/0.129/0.494
		SFE	CO_2	240–480	2.39/0.148/0.407
Carvone/imone	Caraway seeds	Soxhlet	*n*-Hexane	300	0.0163/0.0152
		USAE 69°C	*n*-Hexane	60	0.0145/0.0143
		USAE 25°C	*n*-Hexane	60	0.0172/0.0162
Oil	Rose hip seeds	Soxhlet	*n*-Hexane	180	48.5
		USAE 69°C	*n*-Hexane	60	32.5
		MASE	*n*-Hexane	30	52.6
		SFE	CO_2 + propane	35	66.8

Source: Adapted from Wang, L. and Weller, C.L., *Trends Food Science & Technology*, 17, 300–312, 2006.
Note: MASE, microwave-assisted solvent extraction; SFE, supercritical fluid extraction; USAE, ultrasound-assisted extraction.

efficiency is relatively small and a large number of extractions are necessary to perform a quantitative separation. In addition, the apparatus for continuous LLE automates the process. Since the phases are not shaken, this procedure also helps avoid the formation of emulsions. Continuous extractors using heavier-than-water and lighter-than-water solvents have been described. The apparatus can be assembled to perform extraction alone, or extraction and concentration. One type of continuous extractor operates on the principle of extracting the sample followed by the distillation of the extracting solvent, which is then condensed and passed again through the solution being extracted. The extracted solutes are expected to remain in the evaporation flask. Some restrictions regarding the separated material are imposed in this procedure, including low volatillity and thermal stability. Also, the solvent must be easily distilled [124].

A number of factors are important when choosing LLE as a sample-preparation technique. Chemical equilibria involving changes in pH, ion pairing, complexation, and so on can be used to enhance analyte recovery and eliminate interferences. Other elements of the extraction include the choice of the solvent and of solvent- and aqueous-phase volumes, the time of extraction, the drying of the solvent, the solvent-evaporation procedure, and so on [124]. The LLE organic solvent must possess:

- Low solubility in water (<10%)
- High volatility
- Compatibility with the HPLC detection technique to be used for analysis
- High purity

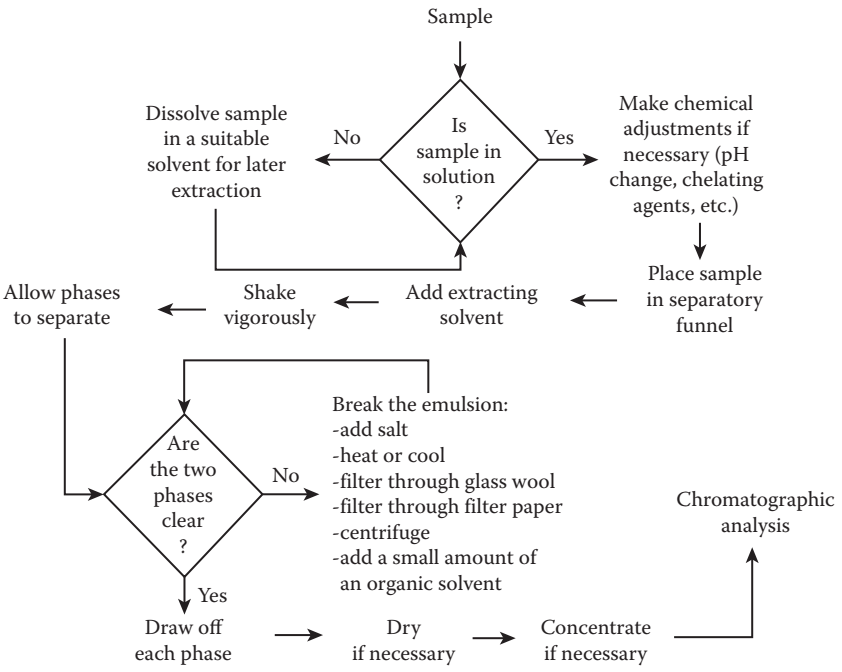

FIGURE 6.6 Schematic diagram of the liquid–liquid extraction (LLE) process. (From Moldoveanu, S.C. and David, V., *Sample Preparation in Chromatography*, Elsevier Science, Amsterdam, 2002. With permission.)

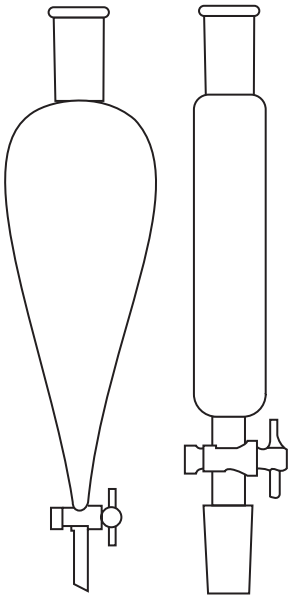

FIGURE 6.7 Liquid–liquid extraction (LLE) apparatus: Separatory funnel.

The organic solvent can be changed to increase the distribution constant K_D. The main selection criterion is low solubility of the solvent in water. A useful aid for determining which solvent pairs are immiscible is a solvent miscibility chart (Figure 6.9). The other factor is the choice of a solvent with a low boiling point for easy removal or concentration after extraction. However, solvents with a very low boiling point may give some problems with controlling the organic-phase volume.

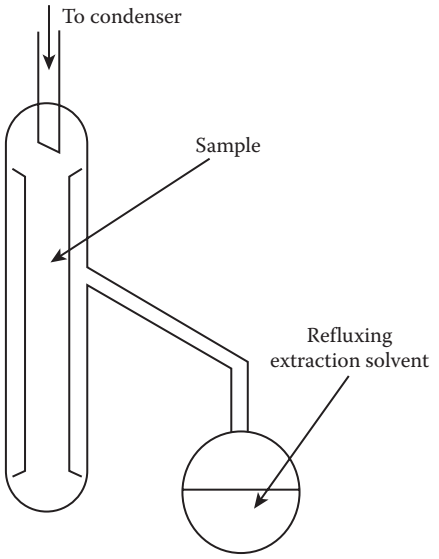

FIGURE 6.8 Apparatus for continuous extraction with an extracting solvent that is denser than the solution from which a solute is being extracted. (Adapted from Snyder, L.R., Kirkland, J.J., and Glajch, J.L., *Practical HPLC Method Development*, John Wiley & Sons, New York, 1997.)

Another parameter is the ratio between the volumes of the solvent and the aqueous solution. The volume of solvent must be adjusted to compromise between efficient extraction and liquid volumes that are convenient to handle and possibly to evaporate when the concentration the organic layer is too low [124].

The distribution constant can be increased, if the analyte is ionic or ionizable, by supressing its ionization to make it more soluble in the organic phase, for example, deliberate extraction of an organic acid from an aqueous solution if the pH of the aqueous solution is lowered (<< p*K*a) so that the analyte is not ionized and will be extracted into the organic phase, leaving more polar interferences in the aqueous phase. The analyte can also be extracted into the organic phase by ion pairing, provided that the analyte is ionized and an ion-pair reagent is added to the organic phase. In order to decrease an analyte's concentration in the aqueous phase, salting out can by used (by the addition of an inert neutral salt to the aqueous phase) [89].

One of the main problems in LLE is the formation of emulsions. It is a drawback, particularly if a solvent of intermediate polarity is used as the extracting solvent, or if the volume ratio of organic solvent to aqueous phase is not sufficiently large. Emulsions may by avoided by changing the phase ratio and can be broken by adding salt or a less polar organic solvent, heating or cooling the extraction vessel, filtering through glass wool or phase-separation filter paper, centrifuging, and so on. Another practical problem associated with LLE is adsorption of the analyte onto the particles that may be present in a sample.

Mutually dissolved solvent can change the relative volumes of two LLE phases. Therefore, it is a good practice to saturate each phase with the other one, so that the volume of the phase containing the analyte can be known, allowing accurate and optimum determination of analyte recovery. Moreover, another disadvantage of LLE is the large volumes of organic solvent used [89,124].

Many biological samples such as plant materials, animal tissue, plasma, and so on are processed by LLE. LLE has been developed for purification of phenolic acids and flavonoids from *Sambucus nigra* L. inflorescence and *Polygonum aviculare*. All methanolic extracts (obtained by Soxhlet, USAE, MASE, and ASE methods) were evaporated to dryness. Dry residues, soaked in boiling water, were cooled in a refrigerator, filtered, and extracted with diethyl ether. Then, the water solution was extracted using ethyl acetate. Ethyl acetate extracts were collected and evaporated to dryness, and then the dry residues were dissolved in methanol and the flavonoids were analyzed quantitatively by

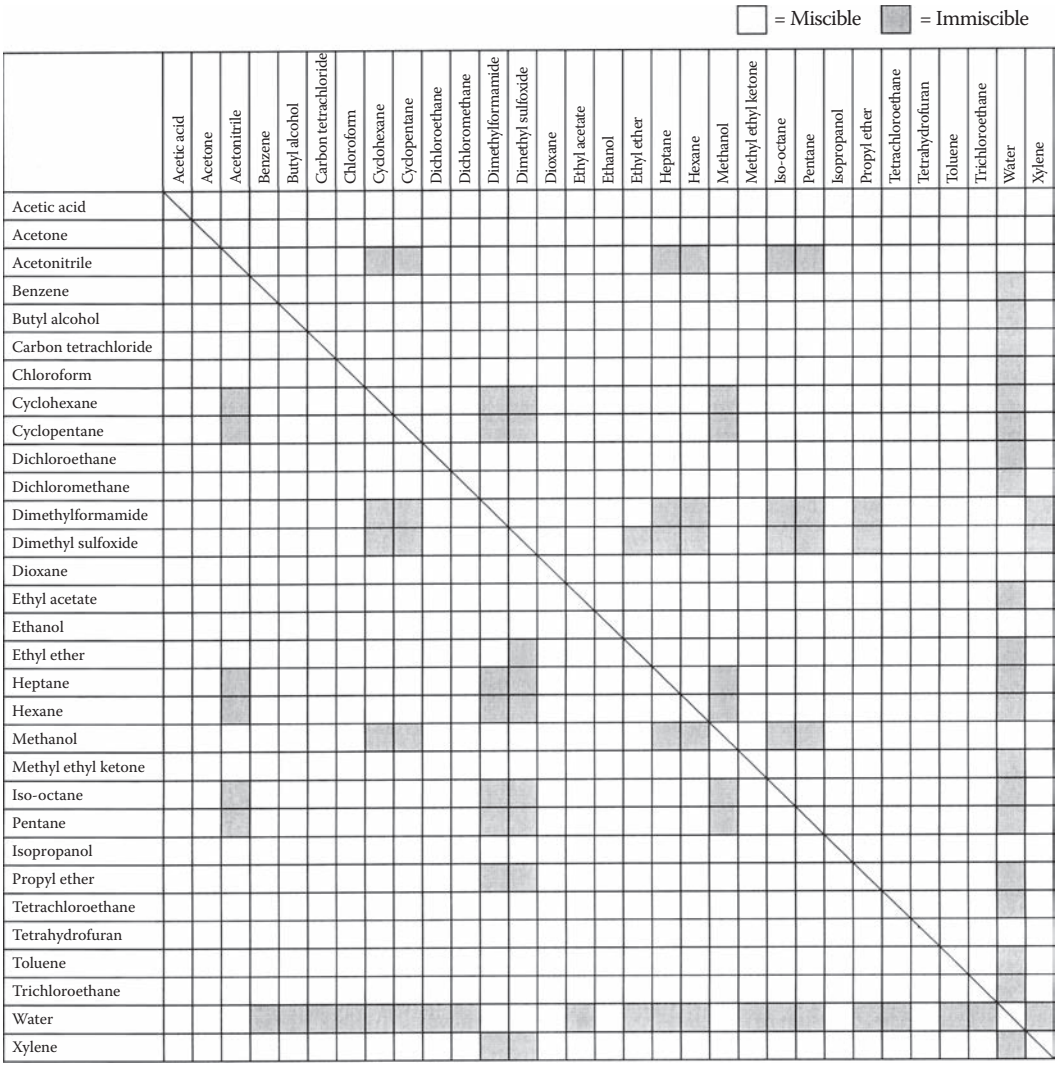

FIGURE 6.9 Solvent miscibility chart.

reversed-phase HPLC (RP-HPLC). Diethyl ether extracts were extracted with 5% aqueous $NaHCO_3$ to transform the phenolic acids into water-soluble sodium salts. The combined aqueous fractions were acidified with 36% HCl to pH 3 and again extracted with diethyl ether. The combined ether extracts were dried with anhydrous Na_2SO_4. After evaporation of the solvent, the dry residues were dissolved in methanol and the phenolic acids analyzed quantitatively by RP-HPLC [92,93].

LLE appears to be the method that repeatedly gives high recoveries of phenolic acids from *Sambucus nigra* L. Recoveries of phenolic acids were determined in extracts fortified with standards and their recoveries ± RSD (relative standard deviation) are the following: for protocatechuic acid, 91.45% ± 1.5%; for *p*-hydroxybenzoic acid, 89.84% ± 1.3%; for gallic acid, 90.21 ± 3.93% [93].

In the case of extraction of tropane alkaloids from *Datura innoxia* Mill., the best purification results were obtained by LLE using dichloromethane from aqueous crude alkaloid extract alkalized to pH 12 [106].

Fujiwara et al. devised instrumentation for on-line, continuous ion-pair formation and solvent extraction, phase separation, and detection for determination of atropine and scopolamine in standard pharmaceuticals [123]. Murphy et al. tested four organic solvents (acetone, methanol, ethanol,

and acetonitrile) in binary aqueous partition for the solvent extraction of 12 phytoestrogenic soy isoflavones from five soy-based matrices with and without the addition 0.1 N HCl in quadruplicate. Special precautions were needed to reduce thermal decomposition. They concluded that acetonitrile was the most efficient extractant [107].

6.3.2 Solid-Phase Extraction (SPE)

SPE is the most important technique used in sample pretreatment for HPLC. The history of SPE dates back at least to the early 1970s, when columns packed with Rohm and Haas XAD resin particles were used to concentrate organic pollutants from water samples [39]. The term *solid-phase extraction* was coined in 1982 by employees of the J.T. Baker Chemical Company [123].

The most commonly cited benefits of SPE in relation to LLE are the more complete extraction of the analyte, more efficient separation of interferences from analytes, reduced organic-solvent consumption and disposal, reduced analysis time, reduced labor (more convenient manual procedures and easier automation), easier collection of the total analyte fraction, removal of particulates, and reduced potential for emulsion formation. Some disadvantages of SPE relative to LLE include the variability of SPE cartridges and the irreversible adsorption of some analytes on SPE cartridges [89,123,125].

SPE is a multistage separation technique providing greater opportunity for selective isolation than LLE, such as fractionation of the sample into different compounds. SPE refers to the nonequilibrium, exhaustive removal of chemical constituents from a flowing liquid sample via retention on a contained solid sorbent and subsequent recovery of selected continuents by elution from the sorbent [123]. SPE is used for removal of interferences from the sample, concentration of the analyte, and desalting. The remaining applications, such as solvent exchange, in situ derivatization, and sample storage, are either seldom used or are less relevent [89]. Retention and elution in SPE can be viewed as a distribution process between a mobile phase and a stationary phase similar to a liquid chromatography (LC) separation performed on a very short column, with a low number of theoretical plates, but involving compounds with very different distribution coefficients [124,125].

In SPE, a liquid sample is added to the cartridge, and a wash solvent is selected so that the analyte is either strongly retained or unretained. When the analyte is strongly retained, interferences are eluted first of all from the cartrige. The target compound is then eluted in a small volume with a strong elution solvent and collected. In the opposite case, where the analyte is weakly retained, interferences are strongly held on the cartridge, and the analyte is collected for further treatment. By either approach, interferences can be removed from the target compounds [89].

Several devices are used for SPE: a cartridge, disk, and coated fiber. The most popular configuration is the SPE cartridges. A typical cartridge may be made of polytetrafluoroethylene (PTFE), polypropylene, or glass. They are generally used once and discarded, because of the danger of sample cross-contamination. Cartridges are available with reservoir volumes of 0.5–10 mL, with packing weights of 35 mg to 2 g. For very large samples, cartridges have up to 10 g of packing and 60 mL reservoirs. A large number of stationary phases are used in SPE (Table 6.5). SPE sorbents should be porous with large surface areas, be free of leachable impurities, exhibit stability toward the sample matrix and the elution solvents, and have good surface contact with the sample solution. The sorption process must be reversible [89,123]. The sorbents are classified as nonpolar, polar, and ion-exchange types. A significant number of sorbents have a porous silica matrix, which is derivatized with specific reagents for obtaining a bonded phase with the desired properties. The other most common polar sorbents are alumina (Al_2O_3), magnesium silicate (Florisil), and the bonded silica sorbents in which silica is reacted with higly polar functional groups to produce aminopropyl [$(SiO_2)_x$–$(CH_2)_3NH_2$]-, cyanopropyl [$(SiO_2)_x$–$(CH_2)_3CN$]-, and diol [$(SiO_2)_x$–$(CH_2)_3OCH_2CH(OH)CH_2(OH)$}-modified silica sorbents. Common bonded phases produced for reversed-phase applications include hydrophobic, aliphatic alkyl groups, such as octadecyl (C_{18}), octyl (C_8), ethyl (C_2), cyclohexyl, or aromatic phenyl groups covalently bonded to the silica gel backbone. The most

TABLE 6.5
Various SPE Phases and Conditions

Mechanism of Separation	Typical Phases	Analyte Type	Loading Solvent	Eluting Solvent
Normal phase	Silica, alumina, Florisil	Slightly to moderately polar	Low polarity, e.g., hexane, chloroform	High polarity, e.g., methanol, ethanol
Adsorption	Cyano, amino, diol	Moderately to strongly polar		
Polar-bonded phase				
Reversed phase	Octadecylsiloxane,	Hydrophobic	High polarity, e.g., water, water + methanol	Low polarity, e.g., hexane, chloroform
Nonpolar-bonded phase	octylsilohexane	Moderately nonpolar	High polarity, e.g., water, water + methanol	Intermediate polarity, e.g., ethyl acetate
- Strong hydrophobicity	Cyclohexyl, phenyl, diphenyl	Slightly polar to moderately nonpolar	High (e.g., water) to moderate polarity (e.g., ethyl acetate)	High polarity, e.g., acetonitrile, methanol
- Intermediate hydrophobicity	Butyl, ethyl, methyl			
- Low hydrophobicity				
Cation exchange	Carboxylic acid	Ionic (ionizable), acidic	Water or buffer	1. Buffer (pH = pK – 2)
- Weak	Alkyl sulfonic acid, aromatic sulfonic acid		(pH = pK + 2)	2. pH value where analyte is neutral
- Strong				3. Buffer with high ionic strength
Anion exchange	Amino1°, 2°-amino	Ionic (ionizable), basic	Water or buffer	1. Buffer (pH = pK + 2)
- Weak	Quaternary amine		(pH = pK – 2)	2. pH value where analyte is neutral
- Strong				3. Buffer with high-ionic strength

Source: Adapted from Snyder, L.R., Kirkland, J.J., and Glajch, J.L., *Practical HPLC Method Development*, John Wiley & Sons, New York, 1997.

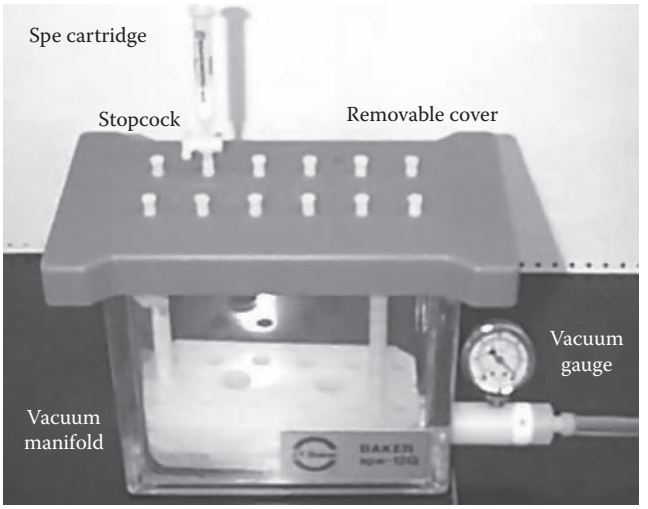

FIGURE 6.10 Solid-phase extraction (SPE) with vacuum technique. (From www.chemicool.com. With permission)

utilized in RP-HPLC is probably C_{18} sorbent. Ionic functional groups, including carboxylic acid, sulfonic acid, aminopropyl, or quaternary amines, can also be bonded to produce ion-exchange sorbents. Moreover, ion-exchange sorbents may by based on apolar polymeric resins. The primary disadvantages of the bonded silica sorbents are their limited pH stability and the ubiquitous presence of residual silanol groups [123,124].

The equipment needed to perform SPE can be very simple. Gravity may be used as the driving force, but flow through the cartridge is extremely slow and impractical for use. Pressure can help provide better contact between the liquid and solid phase and thereby improve the efficiency of the SPE process. When several samples must be processed simultaneously, a vacuum manifold system for processing multiple cartridges at a time is recommended. The vacuum can be obtained by a simple water pump or by a vacuum pump with suitable controls, for example, in the commercial apparatus (Figure 6.10). Regardless of the method used to force the sample solution through the SPE cartridge or other SPE device, the flow rate should not be too fast. Otherwise, there may be an insufficient time of contact of the sample with the stationary phase. For typical SPE applications, a flow rate of 10 mL/min or less is recommended for most cartridges [89,125].

The application of SPE generally involves four stages: conditioning of the packing, sample application, removal of interferences, and, finally, recovery of the analyte. In most cases RP-SPE is carried out, and the analyte initially is retained. In RP-SPE, the packing is "conditioned" by passing a small amount of solvent through the cartridge. The role of the conditioning step is twofold: It removes any impurities and allows the sorbent to be solvated. Methanol is commonly used as conditioning solvent for RP-SPE packing or polar-bonded-phase packing (cyano, amino, and diol). However, silica gel is deactivated by methanol, and intermediate-polarity solvent is recommended for unmodified silica. After the RP-SPE packing is conditioned, the excess solvent should be removed and the cartridge should be purified with a solvent that is miscible with the conditioning solvent and the sample (e.g., water or a buffer). The next step is the application of the sample. The sample must be dissolved in the weak solvent, which allows strong retention of the analyte (Table 6.5). The sample solution should be passed through the cartridge without allowing it to dry out. Afterward, interferences that are more weakly retained than the analyte are washed out of the cartridge, but no loss of analyte occurs. Water or a buffer is often used as the wash solvent in RP-SPE. A small amount of organic solvent may be added to the wash solution. The final step provides for elution and collection of the analyte fraction. This can be achieved with a strong elution solvent. If the analyte is an acid or base, the pH of the sample can be adjusted to suppress analyte ionization

and maximize RP-SPE retention during sample application and removal of interferences. Elution of the substance of interest in the final step can be effected by a change in pH so as to ionize the sample and reduce its retention. After the analyte is collected, the pH of the fraction can be redjusted for optimum retention in the subsequent HPLC separation [89,125].

The SPE method is very often used in sample pretreatment for HPLC. SPE has been developed for purification of phenolic acids and flavonoids from *Sambucus nigra* L. inflorescence and *Polygonum aviculare* (using C_{18} sorbent and polyamide) [92,93] and the taxoids fraction from *Taxus baccata* L. *aurea* (using C_2 sorbent) [126]. In the case of flavonoids—rutin and isoquercitrin—the most efficient purification method for *Sambucus nigra* and *Polygonum aviculare* extracts was SPE using octadecyl sorbent, although the method changes in some degree the composition of extracts with highly polar compounds. The method is characterized by high repeatiblity (RSD ≤ 1.12%). SPE using C_{18} also gives high recoveries of analyzed flavonoids (up to 93.4% for rutin and up to 90.1% for isoquercitrin). Repeatibility and recoveries for investigated flavonoids in the LLE technique were distinctly lower. Moreover, LLE changes the proportions of rutin and isoquercitrin. In the case of SPE of polyamide, the repeatibility is also relatively high (RSD ≤ 2.14%). Recoveries for rutin are also high, up to 93.27%. However, recoveries for isoquercitrin are very low—only about 40%—because polyamide strongly retained more hydrophobic compounds [92].

SPE using a cation exchanger gives good results in the purification of tropane alkaloid extracts from from *Datura innoxia* Mill. In these cases, cartridges filled with cation-exchanger resin were conditioned with 0.005 M/L HCl at a constant and low-flow velocity. The extract, evaporated to dryness and dissolved in 0.05 M/L HCl, was introduced to the column, and eluent was removed. The alkaloid fraction was eluted with a 3:1 mixture of methanol and 10% aqueous ammonia [106].

SPE was compared with matrix solid-phase dispersion (MSPD) to separate phenolic compounds and organic acids from white grapes. The aqueous extracts obtained by ultrasonification were passed through a C_{18} column previously conditioned with MeOH and aqueous HCl (pH 2). The aqueous extracts containing the organic acids were evaporated until dry, redissolved in sulphuric acid, and analyzed by HPLC. Then the retained phenolic fraction was eluted with ethanol and analyzed. Although MSPD could be a useful tool to identify the phenolic and organic acids composition of white grapes, SPE was preferred for obtaining quantitative recoveries of these compounds [127].

6.3.3 Matrix Solid-Phase Dispersion (MSPD)

MSPD is an analytical process, first reported in 1989, for the preparation, extraction, and fractionation of solid, semisolid, viscous, and liquid samples. Its simplicity and flexibility have been cited as contributing to it being chosen over more classical methods for these purposes [128]. This process involves blending a sample with a selected solid phase (such as silica C_{18}, C_8, C_2, Florisil, or alumina), packing the unique extraction/cleanup column, and eluting the target compounds with a solvent. Thus, MSPD is a distinct analytical process for sample disruption and dispersion, which possesses chromatographic characteristics that may selectively elute a single compound or several classes of compounds or completely fractionate the sample matrix components. Therefore, it simplifies the extraction and cleanup steps, reduces sample manipulation, and is faster than conventional techniques. Recently, this method was combined with on-line liquid chromatography [129–131].

The process requires only simple devices (Figure 6.11). It comprises the following steps: A sample is placed in a glass mortar and blended together with a solid support using a glass pestle to obtain complete disruption and dispersion of the sample on the solid support. Sample/sorbent ratios typically range from 1:1 to 1:4. When blending is complete, the sample can be packed into an empty column or on top of a SPE sorbent without any further drying or cleanup prior to elution. The column is often an empty syringe barrel or a cartridge with a stainless steel or polypropylene frit, cellulose filter, or a plug of silanized glass wool at the bottom. A second frit may be placed on top of the sample before compression with a syringe plunger. After successful packing, the sample/adsorbent column is eluted by a stepwise solvent program similar to SPE. The first step is often to wash

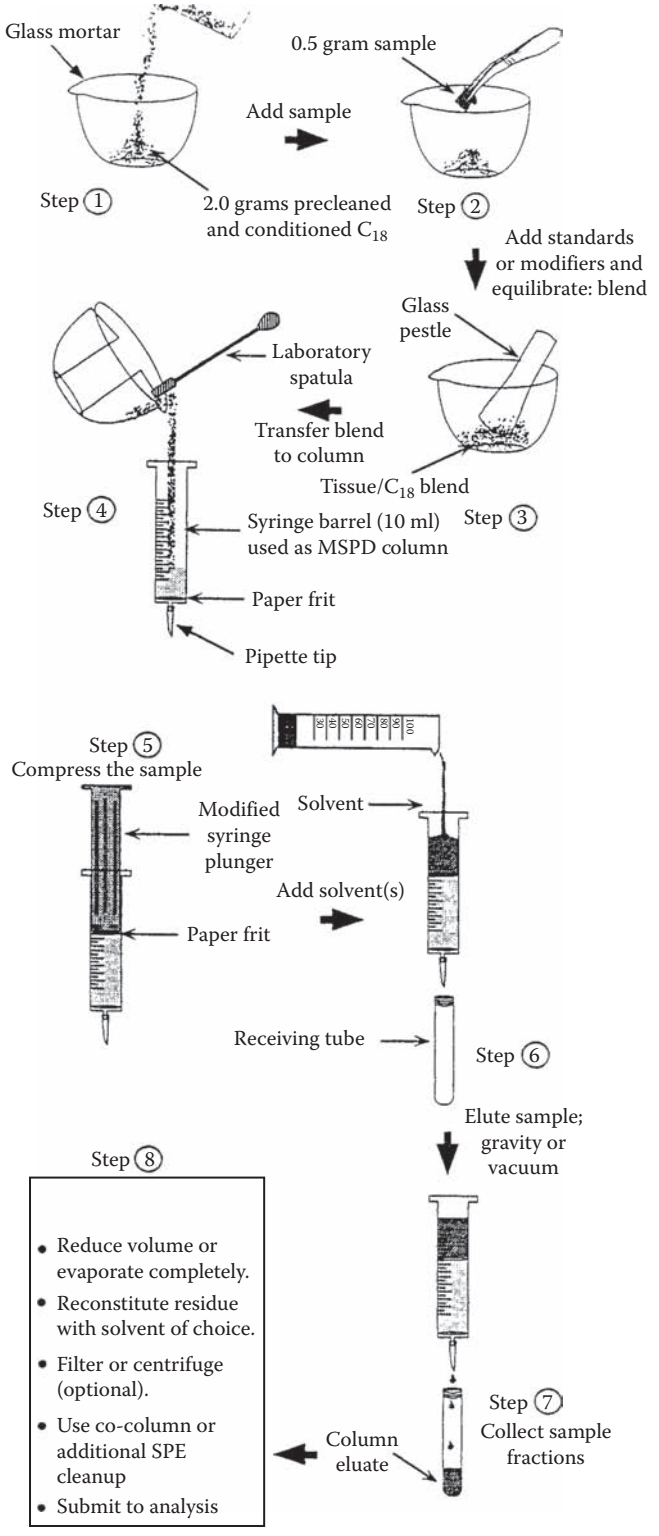

FIGURE 6.11 A schematic representation of the matrix solid-phase dispersion (MSPD) process. (From Barker, S.A., *J. Chromatogr. A*, 885, 115–127, 2000. With permission.)

out matrix interferences like ionic compounds with water. The analytes of interest are then eluted out with suitable solvents and collected in a vial. By using a suitable solvent, the target compounds are eluted out while the matrix components such as polymers and non-polar compounds with lower polarities are still retained in the MSPD column. In this way, the target compounds are simultaneously extracted, cleaned, and enriched [129,132,133]. Most MSPD elutions have been conducted by gravity flow. In some cases it is stated that flow was initiated by the application of pressure to the head of the column similarly to SPE. Many MSPD procedures also employ the use of co-columns to obtain a further degree of fractionation and SPE column, or, as in several recent applications, the sorbent is packed into the bottom of the MSPD column [133,134].

The selectivity of an MSPD procedure depends on the sorbent/solvent combination used. Most methods report using reversed-phase materials, such as C_8- and C_{18}-bonded silica, as the solid support; silica, Florisil, and chemically modified sorbents are used less frequently. For most applications, particles with diameters in the range 40–100 μm are used. The property of the elution solvent is significant since the analytes of interest should be efficiently desorbed while the remaining matrix components should be retained in the column. Naturally occurring compounds are often eluted with polar solvents, such as dichloromethane, alcohols, and hot water. The volume of the elution solvent is also important. It has been observed that for an 8 mL elution of a 2 g MSPD column blended with 0.5 g of sample, target analytes usually elute in the first 4 mL, approximately one column volume. Chemical modification of the matrix (by the addition of chelating agents, acids, bases, etc.) at the time of blending affects the distribution and elution of target analytes from the sample [128,132].

MSPD, like SPE, is a form of chromatography, and the general principles of this technique apply. However, MSPD has been found to be different from classical SPE. For example, MSPD accomplishes complete sample disruption and dispersal into particles of very small size, providing an enhanced surface area for subsequent extraction of the sample, while in SPE sample disruption must be conducted as a separate step. In SPE the sample is usually absorbed onto the top of the column packing material, not throughout the column as in MSPD [128].

The use of smaller sample sizes (about 0.5 g), combined with lower solvent and time consumption, make MSPD competitive with other sample-preparation methods. MSPD has been widely used in recent years for the isolation of a wide range of naturally occurring constituents from different complex plant tissues, providing, in many cases, equivalent or superior results to older official methods using more classical extraction and/or SPE techniques [128,131].

The MSPD procedure was developed for the isolation of phenolic acids from *Melissa officinalis.* The solid phases used for MSPD were C_{18} and Florisil. The sample was mixed with previously cleaned sorbent and *n*-hexane and homogenized. The blend was then transferred into a syringe. Interfering compounds were washed with *n*-hexane and dichloromethane; the syringe was then dried. Phenolic compounds were eluted directly with the polar solvent mixture and the residue after evaporation to dryness was dissolved in methanol–water, filtered, and analyzed by HPLC [132,135].

Also, isoflavonoids from *Radix astragali* were extracted effectively by MSPD-C_{18} and then determined and identified by HPLC–photodiode array detector (DAD)–mass spectrometry (MS). In this method, the column was packed in an extraction layer and a separation layer to increase the chromatographic separation. The results for the fraction collected from two-layer columns indicated that the two-layer method was better than the homogeneous packing method. Several different solvents with good solubility for isoflavonoids, including acetone, ethylacetate, methanol–water, and acetronitrile–water, were investigated. The best result was obtained with 90% aqueous methanol elution [129]. The MSPD method exhibited acceptable reproducibility, recovery, extraction efficiency, and consumption (amount of sample, solvent, and time) relative to conventional extraction techniques such as ultrasonic and Soxhlet methods.

MSPD has been demonstrated to be a suitable technique for the extraction of naturally occurring compounds (for example phenolic acids, isoflavonoids, flavanones, xanthones, asterosaponin) from plant material, a simple alternative to LLE and SPE.

TABLE 6.6
The Application of the Crystallization Process to Purify and Concentrate Mixtures of Active Compounds

Substance Type, Plant Species, and Analyzed Part	Solvent Type	Ref.
Sesquiterpene lactones, phenylpropanoids, *Crepis mollis*, roots	Methanol	[136]
Phenylpropanoid glycosides, *Smilax bracteata*, aerial parts	—	[137]
Friedolanostanes, lanostanes, *Garcinia speciosa*, bark	Methanol, *n*-hexane, and chloroform	[138]
Indole alkaloids, *Ervatamia peduncularis*, leaves, stem bark	Methanol, diethyl ether	[139]
sCoumarins, *Pterocaulon virgatum*, aerial parts	Methanol	[140]
Lycorine-type alkaloid, *Amaryllis belladonna L.*, bulbs	Methanol	[141]
Imidazole alkaloids, *Lepidium sativum*, seeds	Dichloromethane–methanol (2:98)	[142]
Triterpenoids, *Tripterygium wilfordii*, roots	Methanol	[143]

6.3.4 Crystallization

Crystallization is one of the methods of purifying and concentrating substances such as biologically active compounds from plant extracts. This method relies on the exhalation of the active compound in crystal form from a supersaturated or supercooled solution. The aim is to clean up the substance on the basis of differences in the solubility of the substance to be purified and of its pollutants in a specific solvent. This proceeds in several stages. The substance should be dissolved in a small amount of solvent at its boiling temperature and then filtrated or decantated. Then the filtrate must be cooled and crystallized. After that, the crystals should be separated from the solvent. Finally, the substance has to be dried. The crystallization process is often used for concentration and purification of compounds (Table 6.6) [136–143].

6.3.5 Column Chromatography

Column chromatography is frequently applied as a method for concentrating or purifying complicated mixtures such as plant extracts. For example, LC is still used as a sample-pretreatment technique on polydextran gel such as Sephadex [89]. The main difference in comparison with the SPE method is that LC columns are usually considerable larger than SPE cartridges. Besides, the LC columns are usually packed with inorganic packings such as silica, alumina, or Florisil. The method has both advantages and disadvantages. LC makes it possible to work with two LC modes with incompatible solvents e.g., Liquid–Solid Chromatography (LSC) using hexane and Reversed Phase Chromatography (RPC) with water, but it is very time consuming. The trace solutes can be concentrated from the large volume, but then there is the possibility of sample loss (e.g., by adsorption, degradation, oxidation, evaporation). The column effluent can be collected easily, but it is difficult to automate. Besides more difficulties in quantitative analysis or reproducing of results can be observed.

Examples of applications of column chromatography as a method of purification and concentration are presented in Table 6.7 [67,137,141,144–152].

6.3.6 Thin-Layer Chromatography (TLC)

Preparative TLC as a sample-concentration and -purification method can be used when analyzing low-concentration complex samples [153]. Sometimes the extraction and concentration processes must precede TLC to remove impurities that comigrate with the analyte, adversely affect its detection, or distort its zone (e.g., cause streaking). However, an advantage of TLC is the possibility of analyzing crude samples, thereby reducing the number of sample-preparation steps, because the

TABLE 6.7
Application of Column Chromatography in the Preparation of Samples

Substance Type, Plant Species, and Analyzed Part	Solvent Type	Adsorbent Type	Ref.
Labdane triterpenoids, *Aframomum zambesiacum*, seeds	Methanol–dichloromethane (1:1)	Sephadex LH-20	[67]
Annonaceous acetogenin, *Annona cherimola*, stems	Chloroform–methanol (10:1)	Si gel	[144]
Diterpenes, *Gnaphalium gaudichaudianum*	Dichloromethane	Si gel	[145]
Flavonoids, *Lonchocarpus xuul*, stem bark	Chloroform	Sephadex LH-20	[146]
Cyclopeptide alkaloids *Heisteria nitida*, bark	Dichloromethane, gradient elution dichloromethane–methanol (1:1), then ethyl acetate–methanol	Sephadex LH-20	[147]
Phenylpropanoid glycosides, *Smilax bracteata*, aerial parts	Methanol	Sephadex LH-20	[137]
Flavonoids, *Lonchocarpus xuul*, *Lonchocarpus yucatanensis*, leaves, stem bark and root	Chloroform and chloroform–methanol (9:1); hexane–ethyl acetate (8:2); hexane–ethyl acetate (95:5); hexane–ethyl acetate (8:2)	Sephadex LH-20	[148]
Iridoid glycosides, *Harpagophytum prucumbens*, tubers	Methanol–water (6:4)	RP-18	[149]
Triterpenoid saponins, *Harpullia austro-caledonica*, stem bark	Gradient of chloroform–methanol (100:0 to 93:7); gradient of hexane–ethyl acetate (9:1 to 7:3)	Si gel	[150]
Neo-clerodane diterpenoid, *Scutellaria barbata,* whole plant	Chloroform–methanol (10:40)	Sephadex LH-20	[151]
Lycorine-type alkaloid, *Amaryllis belladonna L.*, bulbs	5% methanol in chloroform	Si gel	[141]
Triterpenoid saponins, *Caryocar villosum*, stem bark	Gradient of chloroform–methanol–water (95:5:0–60:40:7)	Si gel	[152]

plate is used only once. Less purified samples, which could not be injected into a GC or HPLC column without breaking off the analysis, are often spotted for TLC; such samples include those containing impurities that become irreversibly adsorbed.

The resolution is improved by optimizing the affinity between sample, solvent, and support. The optimum solvent for separating two or more compounds will maximize the difference in the compounds' retention. Most TLC and preparative mobile-phase systems contain a polar solvent and a chromatographically dissimilar, less polar solvent. As a guide for method development, a substitution in the polar solvent often results in a change in resolution, while a change in the less polar solvent results primarily in a change in R_F of the sample components (R_F, retardation factor in planar chromatography, R_F is the ratio of the distance travelled by the centre of the spot to the distance simultaneously travelled by the mobile phase). The optimum separation of compounds by TLC is usually achieved when R_F values are between 0.3 and 0.5. Generally, adjusting the compound's R_F between 0.3 and 0.5 is done first for a TLC separation. For scale-up to preparative separations, the TLC solvent system's polarity must be decreased to lower the R_F to between 0.15 and 0.35. This R_F range is optimal for a preparative separation, in terms of sample load, resolution, residence time, and solvent usage [154].

Classical layer chromatography can be used for purification and isolation on a micropreparative or preparative scale [155]. Micropreparative separations can be done on precoated TLC layers, with a layer thickness of 0.2 or 0.25 mm, and a stationary phase with an average particle size of about 10 m. For the separation of larger amounts of sample, preparative plates can be prepared. Alumina,

cellulose, and C_2 and C_{18} reversed-phase precoated preparative plates are also available, but silica gel has been the most widely used. The standard layer thicknesses are 0.5, 1, 1.5, and 2 mm. The silica materials have coarse particle sizes (average size ca. 25 m with a distribution between 5 and 40 m).

A higher resolution may be achieved on a thinner preparative layer (0.5–1 mm). The resolution is much more limited on a higher-capacity (1.5–2 mm) layer, owing to the thicker stationary phase. Because of the relatively large average particle size and the diffusion in the higher R_F range, the resolution is limited. When using a laboratory-prepared plate with 15 m particle size or taper plates, this effect can be significantly reduced.

Classical layer chromatography can be used for the purification of a sample when a limited number of zones have to be separated from the compounds of interest. It is essential that the zone of interest should be well-separated from the others, and the location of the zone should be checked carefully before scraping and elution from the stationary phase.

The sequential technique can also be used as an additional purification method for cleanup directly on the plate. This method is a means of improving resolution and reducing separation time by supplying mobile phase to different regions of the plate at different times. The principle of this technique is based on the fact that the mobile-phase velocity is much higher at the beginning of the separation than later. After an initial separation, the layer is carefully dried, and the mobile-phase applicator is placed between two separated zones, whether the same or a different mobile phase is used. The supply of the mobile phase may be stopped at any time in order to transfer it directly to the area of the compound zones to be separated.

Planar LC methods do not compete with column LC for purification and isolation of compounds from a complex matrix. Instead, the two approaches are complementary and together make for successful and rapid separations. In Table 6.8, examples of applications of preparative TLC as a method for the purification or concentration of plant extracts are shown [67,136–139,142,144–147, 156–169].

6.3.7 Membrane Separation

Membrane-extraction techniques can be divided into two main categories, porous and nonporous membrane techniques [170]. In all types of membrane extraction, the membrane separates the sample solution from the acceptor or strip solution, and the analyte molecules pass through the membrane from the donor to the acceptor. If the system is left for a long-enough time, equilibrium between the phases is attained. In most cases of relevance to sample preparation in analytical chemistry, the aim is to transfer as much of the analyte as possible from the donor to the acceptor. To improve this recovery, the acceptor is in many cases flowing, so extracted analytes are removed from the membrane by convection. In some cases, the analytes can be trapped in the acceptor either by a chemical reaction or simply because of a high-partition coefficient, and this will lead to high-enrichment factors. To improve the overall amount of analyte that is extracted, a flowing donor is often used, the sample being pumped on the donor side of the membrane.

Filtration and SPE with disks exemplify great applications of membranes for sample preparation [89]. Moreover, ultrafiltration, reverse osmosis, dialysis, microdialysis, and electrodialysis are examples of techniques that use membranes for concentration, purification, and separation of analytes.

With the exception of filtration and SPE membranes, membrane separation techniques have not been used widely for HPLC sample preparation. Relative to other sample-preparation techniques, membrane separation is slower and less efficient. Compared to SPE or LLE, membranes are less able to concentrate the analyte.

Advantages of membrane procedures over other sample-preparation techniques are:

- The risk of overloading with sample or matrix components is negligible.
- Most membrane processes are performed in a closed-flow system that minimizes contamination and exposure to toxic or dangerous samples.

TABLE 6.8
Application of Preparative TLC in the Preparation of Samples

Substance Type, Plant Species, and Analyzed Part	Eluent	Adsorbent	Ref.
Steryl epoxide, secobutanolide, butanolides, *Machilus zuihoensis*, stem wood	*n*-Hexane–ethyl acetate (10:1); *n*-hexane–ethyl acetate (15:1); chloroform–methanol (18:1); chloroform–methanol (25:1); dichloromethane–methanol (5:1)	Si gel	[156]
Labdane triterpenoids, *Aframomum zambesiacum*, seeds	Hexane–ethyl acetate (8:2; 7:3; 6:4); dichloromethane–acetone (8:2; 6:4)	Si gel	[67]
Annonaceous acetogenin, *Annona cherimola*, stems	Chloroform–methanol (10:1)	Si gel	[144]
Diterpenes, *Gnaphalium gaudichaudianum*	Toluene–diethyl ether (4:1); dichloromethane–ethyl acetate (9:1)	Si gel	[145]
Taxanes, *Taxus Canadensis*, needles	Hexane–ethyl acetate (40:75); hexane–acetone (3:2); dichloromethane–acetonitrile (1:1); hexane–acetone (5:6)	Si gel	[157]
Sesquiterpene lactones, *Lactuca virosa*, roots	Chloroform–methanol (17:3; 4:1)	Si gel	[158]
Flavonoids, *Lonchocarpus xuul*, stem bark	Hexane and ethyl acetate (9:1)	Si gel	[146]
Cyclopeptide alkaloids, *Heisteria nitida*, bark	Chloroform–methanol (99:1)	Si gel	[147]
Triterpene saponins, *Harpullia cupanioides*, stem bark	Methanol–water (65:35)	RP-18	[159]
Sesquiterpene lactones, phenylpropanoids, *Crepis mollis*, roots	Chloroform–methanol (17:3)	Si gel	[136]
Phenylpropanoid glycosides, *Smilax bracteata*, aerial parts	Chloroform–methanol (6:1)	Si gel	[137]
Phenolic compounds, *Pepeormia obtusifolia*, aerial parts	Chloroform; benzene; cyclohexane–acetone (20:1); cyclohexane–benzene (5:1); benzene–acetone (5:1)	Si gel	[160]
Friedolanostanes, lanostanes, *Garcinia speciosa*, bark	petrol–chloroform–formic acid (1:1:0.1); toluene–ethyl acetate–acetone–formic acid (e.g., 88:10:2:1)	Si gel	[138]
Abietane diterpenoid, *Salvia miltiorrhiza*, root	*n*-Hexane–chloroform (4:1)	Si gel	[161]
Polyoxygenated cyclohexenes, *Ellipeiopsis cherrevensis*, aerial parts	Chloroform–petrol–ethyl acetate–formic acid (8:1:1:0.1); chloroform–acetone–formic acid (e.g., 8:2:0.1)	Si gel	[162]
Coumaronochromones, flavanones, *Euchresta formowana*, roots	Acetone–*n*-hexane (1:3); ethyl acetate–*n*-hexane (1:2)	Si gel	[163]
Triterpenoids, *Polygonum bistorta*, rhizomes	Chloroform–methanol (9:1)	Si gel	[164]
Secoiridoid glycosides, *Syringa afghanica*, leaves	Water–methanol (1:1); chloroform–ethyl acetate–methanol (4:2:1)	RP-18 Si gel	[165]
Indole alkaloids, *Ervatamia peduncularis*, leaves, stem bark	Dichloromethane–methanol (97:3)	Si gel	[139]
Triterpenoid saponins, *Harpullia austro-caledonica*, stem bark	Chloroform–methanol–water (60:40:5); chloroform–methanol–water (60:40:5)	Si gel	[159]
Secoiridoid glucosides, *Fraxinus Americana*, leaves	Chloroform–methanol (7:3); butanol–acetic acid–water (4:1:0.5)	Si gel	[166]

TABLE 6.8 (CONTINUED)
Application of Preparative TLC in the Preparation of Samples

Substance Type, Plant Species, and Analyzed Part	Eluent	Adsorbent	Ref.
Phenolic glycosides, iridoid glucoside, *Strychnos axillaries*, barks and woods	Ethyl acetate–benzene–ethanol (4:1:3); chloroform–methanol (7:3); chloroform–methanol–water (70:30:3)	Si gel	[167]
Taxane diterpenoid, *Taxus yunnanensis*, *Taxus cuspidate*, seeds	Hexane–acetone (3:2); hexane–ethyl acetate (3:5); chloroform–methanol (100:5)	Si gel	[168]
Imidazole alkaloids, *Lepidium sativum*, seeds	Chloroform–methanol–ammonia (90:9:1)	Si gel	[142]
Labdane diterpenes, *Leonurus japonicus*, leaves	Hexane–diethyl ether (97:3); hexane–diethyl ether (95:5); hexane–diethyl ether (90:10)	Si gel	[169]

- The use of organic solvents is minimal.
- The flow system permits easy automation.

However, membranes have disadvantages compared to other sample-preparation methods. For example, porous membranes are prone to fouling by particulates or macromolecules; once pores are blocked, flow rates decrease and membrane effectiveness diminishes. In some cases, samples must be pretreated before they can be dialyzed or cleaned up using other membrane techniques.

6.4 HYDROLYSIS OF THE ESTERS, GLYCOSIDES, AND POLYMERIC COMPOUNDS/NATURAL POLYMERS

The hydrolysis of esters, glycosides of plant compounds, and polymeric natural compounds in some cases should be done in order to simplify the analysis. As a result of hydrolysis, simpler compounds of higher polarity are formed. Acidic, alkaline, and enzymatic hydrolysis all have practical applications in the analysis of many substances such as glycosides, esters, and polymeric compounds.

6.4.1 Acidic Hydrolysis

Glycosides of flavonoids, phenolic acids, tannins, triterpenoid saponins, and coumarines mostly undergo the acidic hydrolysis process [171–176]. Complete acidic hydrolysis of phenolic glycosides gave 6,8-di-*C*-glucosylapigenin or vicenin and glucose. These compounds were hydrolyzed with aqueous methanolic hydrochloric acid (1:1) at 100°C for 7 h. Complete acidic hydrolysis of flavonol glycosides afforded kaempferol or quercetin, glucose, and galactose. Each compound was treated with hydrochloric acid (1 h reaction time, in a sealed vial, at 100°C) and, after cooling, the reaction mixture was extracted with diethyl ether. Upon acidic hydrolysis of hydrolyzable tannins, all of the new compounds gave the common constituents gallic acid, ellagic acid, and polyphenolic acids, suggesting that they were ellagitannins. A solution of each compound in sulfuric acid was heated at 100°C for 8 h.

In the case of saponins the hydrolysis was carried out as follows: Each sample was dissolved in dioxane and hydrochloric acid and heated at 100°C for 1 h. The reaction mixture was diluted with water and extracted twice with ethyl acetate. The hydrolysis of coumarin glucosides from *Cruciata taurica* was carried out using hydrochloric acid, and the resulting solution was refluxed for 1 h. The reaction mixture was neutralized and extracted with ethyl acetate to obtain appropriate aglycone.

As indicated in the literature, the disintegration of natural polymeric compounds like polysaccharides, proteins, or pectic polymers can usually be performed by an acidic hydrolysis process [177–179]. Polysaccharide samples were subjected to methanolysis (methanolic hydrochloric acid, 60 h, 80°C), followed by acidic hydrolysis (trifluoroacetic acid, 4 h, 100°C), and the resulting monosaccharide mixtures were analyzed by high performance anion-exchange chromatography.

The amino acid composition from suspension cultures of *Pyrus communis* was determined as their phenylthiocarbamyl (PTC) derivatives, following acidic hydrolysis (solution of hydrochloric acid, 80 h, 110°C) using RP-HPLC.

6.4.2 Alkaline Hydrolysis

In the case of esters of some biologically active compounds, alkaline hydrolysis (phenolic acids, piscidic acid esters, fukiic acid esters, oxanthrone esters, phenylpropanoid esters) can be performed. Acidic hydrolysis is rarely used, mainly in the case saccharic esters [180–184].

Most often alkaline hydrolysis is used in the analysis of some esters. For example, esters of phenolic acids from fruits of *Peucedanum alsaticum* L. and *Peucedanum cervaria* (L.) Lap. were hydrolyzed as follows: Sodium borohydride (to protect against oxidation) and barium hydroxide (to bring the solution to pH 12) were added to the water extract [185]. The solution was boiled under reflux for one hour. The hydrolysate was neutralized using a dilute solution of sulfuric acid, and concentrated sulfuric acid was added to pH 1.5. After filtering the sediment of barium sulfate, the filtrate was extracted with ethyl acetate. The ethyl acetate extracts were dried, and after the evaporation of the solvent, the phenolic acids were obtained.

During the analysis of the piscidic acid esters and fukiic acid esters, alkaline hydrolysis was used. The analyzed compounds were dissolved in sodium hydroxide under a nitrogen atmosphere and stirred for 5 h at 25°C. After that, the solution was adjusted to pH 2.5 by hydrochloric acid, the acidic solution was shaken with ethyl acetate, and, after washing the joined ethyl acetate layer with water, the organic layer was concentrated in a vacuum. The residues were recrystallized from a methanol–water solution.

Oxanthrone esters were taken separately in alcoholic potassium hydroxide and refluxed for 1 h until an aliquot showed the disappearance of the original compound on TLC. It was then cooled and treated with water. The aqueous layer was neutralized with dilute hydrochloric acid and extracted with ether and dried with sulfur dioxide.

Phenylpropanoid esters of rhamnose were dissolved separately in a solution of potassium hydroxide and heated at 60°C for 1 h. To isolate the sugar and the aglycone for further analysis, the reaction mixtures were evaporated to remove methanol and then were extracted several times with chloroform by shaking. In each case, the aglycone separated into the chloroform fraction and the sugar into water. To isolate the aglycone and sugar, the alkaline hydrolysis process should be used, e.g., for secoiridoid and iridoid glycosides [186,187], hydroquinone glycosides [188], or phenylethanol glycosides [189]. The iridoid glucoside syringafghanoside when subjected to alkaline hydrolysis gave monoterpene lactone. A solution of syringafghanoside in sodium hydroxide was stirred for 2 h at room temperature. Hydroquinone glycoside from leaves of *Myrsine seguinii*, Seguinoside E, was treated with sodium hydroxide in methanol at 25°C for 6 h under a N_2 stream.

6.4.3 Enzymatic Hydrolysis

The enzymatic hydrolysis of glycosides by using appropriate enzymes (glucosidases) is also used in analysis of plant extracts [190–198]. Sesquiterpene glycosides from *Dendrobium nobile* undergo enzymatic hydrolysis. Compounds were dissolved in water, and -cellulase was added to the solution and kept at 37°C for 7 days. Diterpenoid glucoside from the aerial parts of *Salvia greggii*, salvigreside A, was also hydrolyzed using enzymes. A solution of salvigreside A in water and β-D-glucosidase from almond was incubated at 37°C for 25 h. The solution was subsequently washed

with EtOAc, and the aqueous layer was evaporated to yield a residue that showed a spot of glucose on silica gel TLC.

In the case of the triterpenoidal glycosides justiciosides A–D, the enzymatic hydrolysis was as follows: Samples of justiciosides were dissolved in methanol. A solution of crude hesperidinase was added. After stirring at 37°C for 1 week, the mixtures were extracted with EtOAc, concentrated to dryness, and then purified. A sample of lignan diglycoside was dissolved in methanol. A solution of crude herperidinase was then added. After stirring at 37°C for 5 days, the mixture was extracted with ethyl acetate and concentrated to dryness, affording isolariciresinol. Two new phenylpropanoid glycosides from the root of *Stellera chamaejasme* were hydrolyzed using β-glucosidase. Compounds were dissolved in three drops of water, and to each was added a small amount of β-glucosidase from almonds. The solutions were kept at 37°C. New flavonoid glycoside from the aerial parts of *Coleogyne ramosissima* was obtained using enzymatic hydrolysis. A solution of the compound in buffer (pH 3.8) and 4% glucose-dimethyl sulfoxide was incubated with hesperidinase at 37°C for 21 h.

Another example of using enzymatic hydrolysis is the isolation of monoterpene glucoside from *Origanum syriacum*. The enzymatic hydrolysis of the substance produced thymoquinol as aglycone. The compound was dissolved in methanol, and a solution of β-glucosidase in water was added. The mixture was extracted with diethyl ether after stirring at 37°C for 2 days. The combined ethereal extracts were evaporated and the residue was purified by HPLC using acetonitrile on a polyamine column to afford the aglycone.

One of the cycloartane glycosides from the leaf and stem parts of *Trichosanthes tricuspidata* gave a new aglycone named cyclotricuspidogenin A by hydrolysis as follows: The glycoside, crude naringinase, and crude pectinase were dissolved in sterilized water. After the addition of a few drops of toluene, the solution was incubated at 37°C for 9 days. The reaction mixture was evaporated, and the residue was purified by column chromatography on silica gel using dichloromethane and methanol to give a new aglycone.

Monoterpene glycosides from the aerial parts of *Solenostemma argel* were hydrolyzed using hesperidinase as enzyme. Compounds were dissolved in methanol, and a solution of crude hesperidinase in water was added. After stirring at 37°C overnight, the mixture was extracted with diethyl ether, and the extracts were evaporated and the residues were purified by HPLC to afford appropriate aglycones.

6.5 PRECHROMATOGRAPHIC DERIVATIZATION

In order to facilitate the analysis, a precolumn derivatization process can be used [89]. There are several advantages for this process in comparison with postcolumn derivatization. Precolumn derivatizaion uses simple equipment and has less restrictions of chemical reaction. It can be performed manually or automatically. There are no time constraints on the kinetics of the derivatization reaction, provided that all the reagents, analytes, and derivatized species are stable. Moreover, the procedure for this process can be used to remove undesired products and sample interferences and, if necessary, to change the sample solvent to be compatible with the HPLC mobile phase.

This method also has some drawbacks, such as the introduction of contaminants and loss of analyte through adsorption, undesired side reactions, possible sample degradation, sample transfer, and incomplete reactions. Also, additional time is required for derivatization, and the added complexity can result in poorer method precision.

Precolumn derivatization is also used in the analysis of plant compounds [199]. In the case of analysis of plant steroids (for example, brassinosteroids) precolumn derivatization is applied. As brassinosteroids lack any chromophore or fluorophore, a major problem for their HPLC analysis concerns their detection. In such cases, precolumn derivatization can be used to produce detectable derivatives. These compouds react with boronic acid, giving appropriate derivatives (bisnaphthaleneboronates, bisphenanthreneboronates, or bisferroceneboronates) that can be detected by UV, fluorescence, or electrochemical detectors and are efficiently separated by RP-HPLC.

Particularly in HPLC, some analytes are more difficult to detect, and precolumn reagents that contain chromophores or fluorophores should be selected to enhance detectability and often decrease interaction problems on the column by reducing the analytes' ability to ionize [200]. Simultaneous analysis of the neurotoxin β-*N*-oxalylα;β-diaminopropionic acid (β-ODAP) and its isomer α-ODAP, as well as other free amino acids, in *Lathyrus sativus* extract was performed using RP-HPLC (C_{18} column) on their para-nitro-benzyloxycarbonyl chloride (PNZ-Cl) derivatives. Samples were derivatized in sodium acid carbonate solution within 2 min at ambient temperature by adding PNZ-Cl in acetonitrile. The PNZ-derivatives were quantified by their UV absorption at 260 nm. PNZ-Cl has been used as a selective, cleavable amino-protecting group in biochemistry. The method has several advantages: On the one hand, the derivatization proceeds rapidly and quantitatively within 2 min at ambient temperature; on the other hand, PNZ-Cl reagent is rather stable to light and air and can be stored in the freezer for several months.

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7 Stationary Phases and Columns in Analysis of Primary and Secondary Metabolites

Fred Rabel

CONTENTS

7.1 INTRODUCTION

Since high performance liquid chromatography (HPLC) began about 45 years ago, many HPLC stationary phases and columns have been introduced by the 50+ manufacturers from the United States and around the world. The number of chemistries (not counting particle sizes or column sizes) that are unique approaches about 800. And this number increases a few percent per year. Although this number might appear daunting, they can be broken down into various subgroups that follow.

Regarding their use, beginning or continuing work with these packings today is easier than in the past. There is a rich history of publications on HPLC in the phytochemical area, so this can help one begin with the experience of other researchers. Thus, by going to the literature and texts such as this one, much time is saved in the initial pursuit of a satisfactory separation.

This chapter describes the major modes of chromatography and the stationary phases available for use in phytochemical separations. All of the commonly used supports can be modified with chemical species by a variety of methods. Figure 7.1 illustrates the general reaction that would occur with some chemically reactive group on the stationary phase or support, being reacted with a complementary reactive group on the bonding reagent. The spacer shown is also important to allow more complete attachment of the bonded phase or to allow better interaction with the solutes to be separated later. Its length is changed as needed when optimizing the bonding process.

7.2 ADSORPTION MODE

Most of the separations of phytochemicals in the literature have been done on pure silica gel columns or thin-layer chromatography (TLC) plates. This is particularly true for separations done before the advent of bonded-phase silicas. Since the adsorption mode works on the basis of the differences in the polarities of the species present (with the least polar eluting first and the most polar eluting last), it has served the analytical chemist well over the years.

7.2.1 Silica Gel

Basic silica gel has the formula SiO_2. The active sites on silica gel that control the separation are, however, the silanols, SiOH. By weight, the percentage of hydrogen is so small, however important, that it does not appear in the chemical formula.

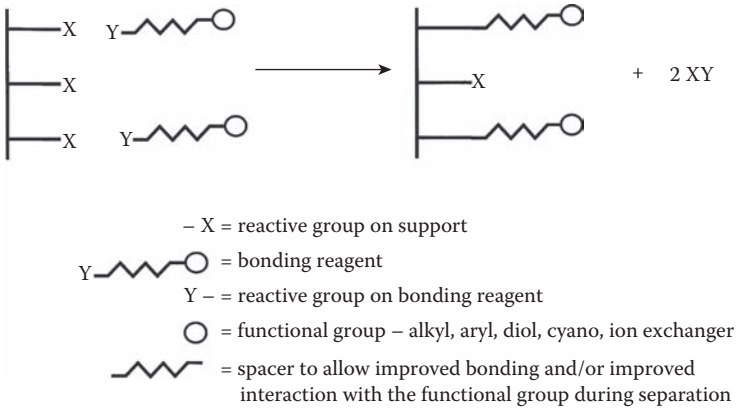

FIGURE 7.1 General bonding scheme for HPLC packings (silica gel or polymers).

TABLE 7.1
Comparison of Silica-Gel Pore Sizes, Surface Areas, and Molecular-Size Limits

Pore Diameter (nm)	Surface Area (m^2/g)	Suitable Mol Weights
5	~500	< 2000
10	~300	< 5000
30	~60	< 35,000
100	~30	< 100,000
400	~10	> 250,000

Source: This table was constructed from information gathered from several manufacturers.

Depending on the way the silica is made, there is a balance between the pore size and the surface area. As can be seen from Table 7.1, these properties are inversely related. As the pore size increases, the surface area decreases. A silica with larger pore size will have a smaller surface area and fewer silanols, thus would be less active. This would be seen in shorter retention times in an HPLC column or on a TLC plate. It would also result in a smaller mass capacity should scale up be done. Finally, the smaller surface area would mean less bonded phase could be attached, also leading to less retention and less loading capacity on larger-pore-size packing with the same bonded phase.

Consideration of the pore size is important when choosing a suitable HPLC column packing to separate sample components with larger-molecular weight. Table 7.1 also shows the approximate molecular-weight species that can be separated on the various pore-size supports.

Silica gel has many advantages from the manufacturer's point of view. It is relatively inexpensive to make a good silica gel product. It is also ideal for bonding since the addition of many bonded phases is possible by simply attaching organosilane derivatives to the available silanols.

The one classic disadvantage is that silica gel can dissolve under certain conditions, thus damaging silica gel packing in an HPLC column. This occurs whether the column is pure silica gel or a bonded silica gel. This is because any bonding chemistry, even if the product is endcapped (a secondary attachment of a smaller organosilane), leaves a significant number of silanols on the surface. These are the points of attack for many mobile phases.

There is no problem if the mobile phase contains only mixtures of organic solvents or organic solvents miscible with water. However, if any ions are present—such as an added buffer, even at pH 7—the dissolving can be significant. The higher the ionic strength, and the higher the pH, the faster this attack occurs [1].

Ionic components in the mobile phase accelerate the formation of silicate ion from the silanol sites. Under average conditions, with mobile phases containing only moderately basic pHs (pH 7–8) and ionic strengths (0.005 molar salt or buffer), columns can be used for a couple of months before any significant change would be seen in the chromatogram. The signs of deterioration of the packed silica bed are peaks that are much less symmetrical and broader, indicating a loss of column efficiency [2].

The older silica gels were made by dissolving sand in an alkaline solution, followed by filtration, centrifugation, and reprecipitation with an acid. By varying the acid strength, rate of addition, stirring, and heating, the pore size and the surface area of the silica can be controlled. These are now referred to as **Type A** silica gels. With this type of manufacture, the silica contained more heavy metals and sodium and potassium ions, some of which can interact with selected solutes through ionic forces or complexation [3].

The result could be tailing or other peak artifacts, which are often traced to these various impurities. Often it was necessary to use additives to eliminate the artifact—usually poor peak shape—or to minimize it. The additives might be triethylamine, diethylamine, or another weak base.

In the 1990s manufacturers began to use an alternative way to make their silicas, which were mostly spherical at this point in time. They would make a volatile organosilane, like tetraethyoxysilane, from the silica gel. This could be distilled, leaving behind nonvolatile impurities like heavy metals. This pure organosilane was then hydrolyzed to make a silica sol, which could be formed into the spherical particles. This newer, purer silica is referred to as **Type B** silica gel.

The advantage of this newer silica gel soon became very apparent. The components of most sample mixtures generate peaks that are more symmetrical, and, as an added advantage, these silicas (and their bonded counterparts) are much more stable in higher-pH (11–12) ionic mobile phases. They are equally stable in lower-pH (4–8) ionic mobile phases.

Most manufacturers now use only this silica gel in the preparation of all their column packings. The combination of the use of Type B silicas for bonding, manufacturers' knowledge of more stable and reproducible chemistries, and the availability of these in smaller-particle sizes (discussed in the following) are all reasons why the newer bonded-phase packings (made in the past 10 years) should be used on any new separation being developed.

7.2.1.1 Irregular

The original silica gel used to manufacture HPLC columns was derived from the classical larger irregular particles used in standard column chromatography. It was sized to a narrow range with a mean particle diameter of 10 ± 2 μm or smaller. It was simple to produce but needed to be packed under high pressures to get a suitable packed bed that would perform well and reproducibly.

7.2.1.2 Spherical

About five years after the introduction of HPLC columns made with irregular silica, manufacturers began to make spherical particles. During the manufacturing process, surfactants and other chemicals were added to the silica sol of microspheres. These microspheres were allowed to agglomerate into larger-spherical silica particles. If necessary, they were screened or air-classified to the desired particle size range. Over the years, the average particle size of the HPLC columns being used by most chromatographers has decreased from 10 to 5 μm, to 3.5 μm, and, in the ultra-HPLC (UHPLC) system, even to sub-2 μm particle size (presently with average particle diameters of 1.8, 1.7, or 1.6 μm).

As already indicated, there is always a ± range to the average particle size, since it is neither possible nor economical to produce a packing of a single diameter. Thus, it is possible to see packings claiming to be the same particle size and range, but because the particle size distribution is skewed, their columns give different backpressures. This assumes the same packing technique is used to produce them.

7.2.1.3 Monolithic

Although attempts had been made for many years to grow the same basic silica sol into a continuous porous rod, it was not until 2001 that E. Merck in Darmstadt, Germany, commercialized the work of Japanese researchers with whom they had been collaborating. They are known by the brand name, Chromolith. E. Merck also licensed Phenomenex to make and sell the same products, with their brand name Onyx.

The Chromolith columns have unique properties that packed-bed columns do not. They are more porous (about 81% versus 70% for packed beds), hence develop much less backpressure initially and through use. They give efficiencies of up to 100,000 plates per meter, similar to 3.5-μm packed beds. The Chromolith columns are made with Type B silica gel, hence are stable at pHs up to 12. They

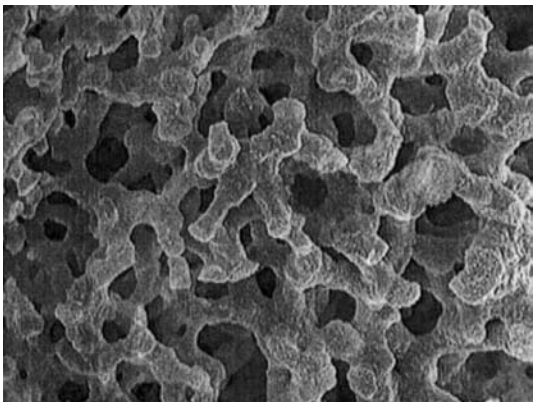

FIGURE 7.2 Scanning electron microscope (SEM) photo of the Chromolith monolithic silica gel structure.

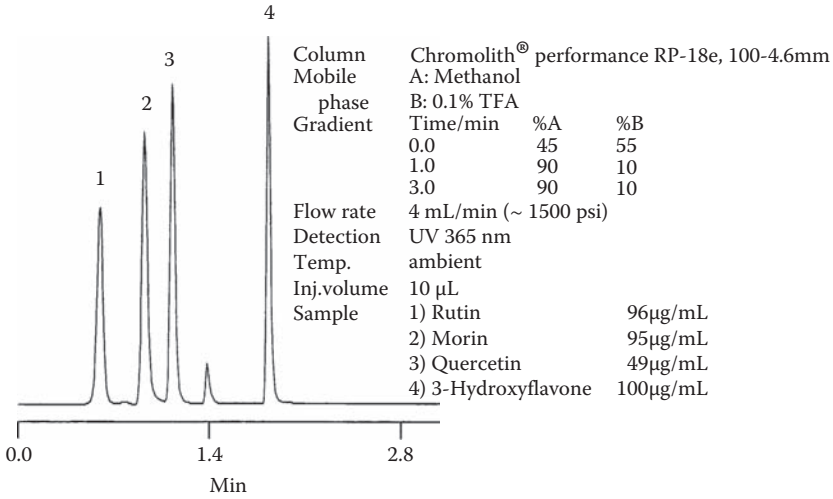

FIGURE 7.3 Separation of flavonoids on a Chromolith column showing typical low backpressure, speed, and resolution.

are available as plain silica but also as bonded reversed-phase columns, discussed in Section 7.3. Figure 7.2 shows the structure of the monolithic silica, and Figure 7.3 illustrates the low backpressure, speed, and resolution of a typical separation done with a Chromolith monolithic silica column.

When doing fast HPLC on packed particulate columns, one problem arose. As the particle size of the packing decreased, the backpressure increased. When the flow rate (or linear velocity) is increased, two things occur. The backpressure reaches the limits of the pumping system (to shut it off), and the efficiency decreases. The monolithic silica gel, with its more open bed, solves the previously mentioned problems. They can be operated on a standard HPLC system at high-flow rates, and they lose little efficiency when operated at these high-flow rates [4,5].

7.2.2 Silica Hybrid

When producing the silica sols for eventual formation of spherical particles, it is possible to introduce organosilanes to form an organosilica particle. These combination particles are referred to as hybrid silica gels and were first commercialized as Xterra and XBridge by Waters Corporation

(Milford, Mass.). They, like any other support materials, introduce a slightly different character to the separation and also have an extended life, being more resistant to the dissolving mentioned earlier [6].

7.2.3 Alumina

Irregular aluminas have been used as column chromatography packings for many years, along with silica gel. As with the silica gels, they could be made into smaller-particle size ranges for use in HPLC. Their advantage was that they could be used with high-pH (to pH 14) mobile phases without dissolving the support.

Aluminum oxide (alumina) exists in many forms: α, χ, η, δ, κ, θ, γ, ρ. These arise during the heat treatment of aluminum hydroxide or aluminum oxy hydroxide. The most used for chromatographic applications is γ-aluminum oxide. Its surface can be made acidic, basic, or neutral through chemical manufacture or treatment. Alumina has many different surface characteristics involving its Al, O, and H ions. This gives many sorption possibilities depending on the mobile phase [7]. When using either an irregular or spherical version, check with the manufacturer on the details of their alumina HPLC packings, most importantly the surface pH, to prevent unwanted absorption of components of the sample.

7.2.4 Other Inorganic Oxides and a Carbon Support

Although a few other inorganic oxides such as zirconia and titania have been used in HPLC columns on an experimental basis, only zirconia has been commercialized. Because these oxides are of limited use, there are fewer references on them pertaining to separations in the field of phytochemicals. Their use is recommended when high-pH mobile phases are required, since they are more resistant to dissolving. Reference 8 is an excellent review of how these inorganic and organic supports (also discussed in Sections 7.2.1.3 and 7.2.2) are synthesized.

A porous graphitic carbon support has been available for a number of years. It can be used in the reversed-phase or normal-phase mode. It, too, is stable from pHs of 1 to 14 in a wide range of solvents and conditions. Unlike other less-known supports, it has found modest use, and a number of applications of phytochemicals can be found. It is branded as Hypercarb (Thermo Scientific).

7.3 REVERSED-PHASE MODE

Because of the solubility of many biological compounds, whether plant or animal, in aqueous, aqueous–methanol, or aqueous–acetonitrile solvent(s), separations of many classes of compounds have been accomplished on reversed-phase bonded silica gels. Today, probably 80% of all separations done or reported in the literature are separated on such phases.

7.3.1 Silica Gel Bonded with Hydrophobic Phases

Through many routes, each HPLC column manufacturer began to bond a hydrophobic phase to silica gel from the very beginning of the production of packed HPLC columns. Although many separations had for years been accomplished on pure silica gel columns, early work showed the advantage of such phases that were simply "coated" on the silica gel, by putting the hydrophobic phases directly into the mobile phase. This was not ideal for analytical work, since ensuring the same level of coated phase was problematic. Any change in temperature, flow rate, or the ratio of water to organic solvent changed the percentage of the phase coated on the silica gel. Obviously, by bonding these phases, stable reversed-phase packings could be made for use in HPLC columns.

Initially, the most often used bonding chemicals were tri- or difunctional organosilanes, which had been available for many years for the manufacturers of silane bonding reagents. The leaving groups were either chloride or hydroxide. Although each could be used when bonding to silica gel, the cleanest reaction mixtures were with hydroxide, since on hydrolysis of the reaction mixture, the result was water.

The bonding reagents normally have only one major group, such as a C18 or a phenyl. The other attached groups are smaller, such as methyl or ethyl groups. This allows more silane reagent to bond, since such reagents are less bulky. The smaller spatial area of these reagents allows better accessibility to available silanol sites for bonding. More recently, the attached smaller groups might also contain more polar groups, like an ether, amide, carbamate, or urea group. This type of bonded phase is called a "polar embedded alkyl phase" and imparts better stability to the bonded phase during use with high-water-content mobile phases. Often these packings are referred to as aqueous stable, with an *aqua*, *aqueous*, or *hydro* in their name or description [9]. See Section 7.3.1.6 concerning endcapping with reagents containing similar polar groups.

The theory behind the "aqua" phases is better stability in high-water-content mobile phases, as this promotes easier solvation of the hydrophobic bonded phase in such mobile phases. This type of phase can also be made with a special endcapping reagent, discussed later in this chapter. Each manufacturer has developed a number of proprietary bonding technologies in their attempt to achieve the "perfect" bonded phase for all applications.

If the bonding reagent has two or three leaving groups, it is possible that bonding might occur twice with each molecule if there are two silanol groups in close proximity to one another. Or, if this cannot occur, only one of the two or three leaving groups bonds, and on hydrolysis, the SiOH groups can cross-link near the surface of the silica gel, giving extra support to the bonded phase. Because of these possibilities, bonded phases made with these reagents are referred to as "polymeric" reversed phases. Usually these are the older reversed-phase packings made by most companies over 20 years ago.

In the past 20 years, however, most manufacturers have used bonding reagents with only one leaving group. This gives more reproducible results from the manufacturers' and users' point of view. These reagents can bond with only one silanol and are referred to as "monomeric" bonded reversed phases.

It might be assumed that either a monomeric or a polymeric C18, with the same carbon load, would give the same separation results. This might be true for simple mixtures, with three or four components. However, with more complicated mixtures, say polyaromatic hydrocarbons or a bioderived sample, the separations on each are very different. The elution order and resolution of various peaks are unique, and different mobile-phase ratios or gradients are required for optimization.

There is no way to generate the same separation on both types of bonded phases, regardless of the mobile-phase combination used. This has led to some special uses for monomeric or polymeric C18 phases. Two that can often be found in the literature are the separation of polyaromatic hydrocarbons and the separation of corticoids, which require a polymeric phase for the most complete separation [10,11]. If a C18 column is not labeled as polymeric or monomeric, then checking with the manufacturer can confirm which it actually is.

Finally, after the major group is bonded, there will be more silanols remaining. These might cause problems with a separation, so a second bonding step is often used to try to bond remaining silanols. This second bonding is called *endcapping* (Section 7.7). Endcapping is done with the smallest possible reagents to allow the greatest accessibility to the silanols throughout the silica. Most often this reagent is trimethylmethoxysilane [$(CH_3)_3SiOCH_3$]; however, in the past 8 years, other silane reagents with polar imbedded groups have also been used [9].

Because of all the variety of organosilanes used for reacting with a support, and the variety of conditions, the carbon% that results can be from a couple percent to as much as 20%. Generally, the

more carbon loading on a packing, the greater the retention time, and the more organic solvent is needed in the organic-water mobile phase to decrease the retention.

Even if more than one endcapping reaction is done on a silica gel, it is impossible to bond all the residual silanols. Thus, some solute–silanol interactions might occur, giving a misshapen peak. Unfortunately, this can never be predicted. One solute can give a perfect-shaped peak, but a very similar one will show tailing. The silanol content of bonded phases is discussed in Section 7.3.1.7.

It is recommended that once a specific brand and chemistry are chosen that the user purchases at least another lot of this HPLC column from the manufacturer. Test this column with the protocol developed for assurance that the manufacturer has control over its reproducibility.

7.3.1.1 Bonded C18

Bonded C18 phases were the first onto the market, in the form of loose packing or as prepacked HPLC columns, first in irregular 10 μm packing size and later in the smaller sizes previously mentioned. The irregular 10 μm bonded C18 is the most used bonded reversed phase and the most cited in references, since it was specified in the early LC methods and written protocols.

Every manufacturer has their own silica-manufacturing process (whether or not they, indeed, do manufacture the silica gel—many buy it from primary sources). This might include any number of steps from heating to various temperatures, washing it in acids or bases, or other processes. Each combination eventually leads to different bonding characteristics on the silica gel surface.

Likewise, the bonding chemistries and conditions are quite different from one manufacturer to another. Thus, although the names might appear to indicate the same bonding chemistry, their selectivities are quite different from one another. It is impossible to simply switch to another manufacturer's column, or one from the same manufacturer with a different brand name, and get the same results. Often a lengthy reoptimization process is involved. For this reason, the column cited in an article is the one that is most often used, in spite of the phrase "or equivalent" that so often follows the statement of the column used. Finding that "equivalent" column is very difficult, so most chromatographers try to use only that which was specified in the original paper or protocol. Unfortunately, this leaves the chromatographer with a method that is useful but not as robust as it could be using the newer, more stable, and faster HPLC packings and columns available today.

To simplify the finding of "equivalent" or "nonequivalent" HPLC packings, the U.S. Pharmacopeia (USP) has added two C18-column-comparison tools to their Web site. One is based on a standard mixture produced by the National Institute of Standards and Technology (NIST), referred to as the USP approach. The second is based on a set of 150 standards used by Lloyd Snyder and coworkers to characterize C18 bonded phases, referred to as the Product Quality Research Institute (PQRI) approach. The details of the theory and use of these tools can be found on the USP Web site at http://www.usp.org/USPNF/columnsIntro.html.

7.3.1.2 Bonded C8

Often, after introducing the bonded C18 packings and columns, the manufacturers made the C8 version, too. With some compounds, the C18 phase is too hydrophobic, and a shorter-chain-length bonded to the silica gel gave improved results. For many compounds, it also meant the organic portion of the mobile phase could be reduced for the same retention time as found on a C18 column. This is because there is less carbon on the silica gel, hence less retention.

Many separations can benefit from going to a C8 bonded phase, since the separation time is shorter, the solute mixture might be more soluble in the more water-rich mobile phase, and the equilibration times are faster after a gradient or when initially setting up a column for ion-pairing work.

7.3.1.3 Bonded C4–C2

Even shorter-bonded phases are available from a few manufacturers. Many manufacturers offer a C4 or a propylcyano, which would be slightly more polar. Fewer manufacturers offer C2 or Trimethylmethoxysilanol (TMS) bonded packings.

A problem with using these short-chained bonded reversed-phase columns is that under acidic conditions (pH 2 or less), the shorter-chain-length phase will be cleaved more readily than a C8 or C18 bonded support. Thus, very-short-chain bonded phases are suitable for use only with methods requiring only mobile phases with a neutral or slightly basic pH [12].

7.3.1.4 Bonded Phenyl or Diphenyl

Bonding phenyl groups as a single group or two groups on a silane reagent has been available for many years. Originally, the thought was to promote the π–π interaction of the aromatic ring as a mechanism. This can, of course, happen, but it can also be considered as a different hydrocarbon phase with different solvation, leading to a different selectivity. Separations on such a phase can be attempted when the solutes being separated have phenyl groups that might be amenable to π–π interactions. A few manufacturers have made a fluorinated phenyl bonded phase that improves the separation of taxol and related compounds [13].

7.3.1.5 Bonded with Fluorinated Phases and Longer-Chain-Length Phases

Other aliphatic hydrocarbon chain lengths and fluorinated versions of the C18, C8, or phenyl groups have been bonded to silica gel. Only a few of these have been commercialized or found use. In general, fluorinated phases are more polar than their hydrogen-containing versions. The fluorinated phenyl phase for taxol separations has already been mentioned [13]. Of the bonded longer-aliphatic chain, also discussed in the preceding, only the C30 bonded phase has found use, for the separation of carotenoids and lycopene [11].

7.3.1.6 Bonded with Mixed Hydrophobic: Hydrophilic Phases

Although so many packings have been made with only hydrophobic bonded phases to give the reversed-phase character to separations done on them, problems sometimes arose with their use. With mobile phases containing a lot of water (> 85% water or buffer), the performance on some of these packings seemed to indicate that the bonded hydrophobic phase, the C18 or other, might be collapsing. As peaks that were well separated with symmetrical shapes began to merge and become mis-shapen as more samples were run. Often this was a temporary setback, since resolvation with a more organic-rich mobile phase seemed to bring the bonded phase back to its original state. This was, however, not an ideal situation, since one had to stop in the middle of the sample runs to regenerate and resolvate the HPLC packing.

To avoid this, some manufacturers began to bond more polar groups mixed in with the original C18 or C8 or to add some polar groups during endcapping. This variation in the bonding chemistry of reversed phases was discussed in the preceding. Look for mention of "aqua" in the name or a description indicating the phase's stability under high-water-content mobile-phase use.

Two brand names of such packings are Atlantis (Waters Corp., Milford, Mass.) and Neptune (ES Industries, West Berlin, N.J.), which clearly indicate their purpose.

7.3.1.7 Silanol Content on Bonded Reversed-Phase Packings

Many manufacturers claim that there are few or no remaining silanols on their bonded reversed-phase packings. This is due to their bonding process or bonding reagents, or multiple endcapping reactions their products undergo.

On the normal silica gels used for bonding today, there are about 8 μmol/m^2 of silanols available on and in the silica gel. If the data for any of the modern bonded phases are examined, however,

the bonding takes up no more than 4 $\mu m/m^2$ of available sites. This means that only about half of the silanols were able to be bonded with the initial reagent (C18 or C8, etc.) or the endcapping reagent [1].

Thus, although unfavorable interactions with Type B silica gels are less common than with older bonded phases on Type A silica gels, there can still be interactions between certain end groups on the species being separated and residual silanols, causing peaks that tail or are broadened. The solution is to either change to another column or find a pH, buffer, or additive that will minimize or eliminate the problem peak(s). These might be noted in mobile phases that contain an organic amine (diethylamine, triethylamine).

7.3.2 Silica Gel Coated with Hydrophobic Phases

One manufacturer has made a silica gel with a polymeric coating. This was done to impart greater resistance to any dissolving effect of the mobile phases, as already described. Although a possible solution to this problem, any polymeric phase, whether it is coated on a support or the support is totally polymeric, suffers from slow mass transfer through the matrix and low efficiency. Equilibration from one mobile phase or pH to another is very slow. Very few references are found in the literature using a silica gel coated with a hydrophobic phase.

7.3.3 Alumina Coated with Hydrophobic Phases

Alumina has also been coated with a hydrophobic phase by a couple of manufacturers. This support is, of course, pH stable to 14, but it does not have any stable OH groups onto which a phase can be bonded.

To impart a reversed-phase surface to this more pH-robust support, a polybutadiene polymer has been applied to the spherical surface. This works well, but again low efficiency is the result because of slow mass transfer. This coated alumina has also found modest use for phytochemical separations, so it does turn up in the separations literature.

7.3.4 Polymer Supports

A number of totally porous polymeric supports have been made for chromatography for almost 50 years. They are the basis of many original LC applications, even before higher-efficiency supports were made. The supports are copolymers of styrene-divinylbenzene, polymerized methylacrylate, acrylamide, or others. They can be used for separations without further derivatization or can be derivatized during their manufacture with C18, C8, C4, phenyl, or other intermediates so that ion exchangers can be formed throughout their subsequent matrix. Their main advantage is their resistance to high pHs. Their disadvantage is slower mass transfer and slower equilibration when conditions are changed.

Unless they are highly cross-linked, they have one other disadvantage. They can expand or contract with different solvent combinations, so high backpressure (when the polymer is expanded) or broadened peaks (when the polymer is contracted) can result. Often these packings are available in hardware with adjustable end fittings that allow them to be moved to accommodate the expansion or contraction of the column bed. If their use is considered, read the complete operating instructions from the manufacturer before proceeding.

7.4 NORMAL BONDED PHASE MODE

Although most separations today seem to be done on silica gel bonded with reversed phases, the plant world is filled with mixtures from nonpolar to polar components. In the past, most of these mixtures were separated on silica gel TLC plates. Today, however, more reproducible separations

of phytochemicals are found using the polar bonded versions. This is most likely due to decreased silanol interactions with moisture that gets into the mobile phase or is introduced when the samples are injected. Often it is possible to just change the packing from a silica gel to a polar bonded phase on silica gel to accomplish the same separation, with only slight tweaking of the mobile phase.

7.4.1 Silica Gel Bonded with Hydrophilic Phases

The bonding of the polar phase is done in a similar manner to that of the bonded reversed phases. A silanol reagent with the single functional group and two methyl groups is often used to ensure better bonding with less steric hindrance. It should be noted that because of the reactivity of many of these polar functional groups, the polar bonded phases are not endcapped. Since the bonded groups are polar, the remaining silanols only contribute in their own way to the final selectivity of the bonded-phase packing.

Although silica gel bonded with hydrophilic phases has been available since HPLC packing were first made, their use accounts for only about 15% of the packings used. Thus, fewer references to their use in phytochemical separations are found.

7.4.1.1 Silica Gel Bonded with Cyano (CN)

The major group used for bonding a cyano group is the propylcyano: CH_2-CH_2-CH_2-CN. It is very similar to a bonded C4 phase, only slightly more polar. Like most of the polar bonded phases, it has been used or tested occasionally but is not in wide use.

7.4.1.2 Silica Gel Bonded with Amino (NH_2)

Again, for bonding, the propylamino group (CH_2-CH_2-CH_2-NH_2) is used on the bonding reagent. This bonded phase is particularly unique since it is a reactive group that can be used as an intermediate to link any number of other chemical groups. It can also easily be converted to a quaternary form, which might be less useful for a separation previously done with little problem. This phase can be converted back to its base form by washing it with 20 column volumes of a 0.01M NH_4OH solution after it is solvent conditioned to water. This can be converted later to the mobile phase used in the separation. Such solvent change over is described in Section 7.12.8.

This phase is used quite frequently for carbohydrate separations using acetonitrile–water mobile phases in the range of 70:30. Although it might appear as if a reversed-phase separation is being done, it is actually a normal-phase separation, as more water makes the carbohydrates elute faster.

7.4.1.3 Silica Gel Bonded with Diol—2 (OH)

The reagent used to make this phase is derived from glycerol (CH_2-CHOH-CHOH) so that two alcohol (–OH) groups are present. As with the bonded NH_2 phase, this bonded phase is a reactive group, so use with mobile phases that contain alcohols should be avoided, since ester formation is possible on these groups. Once such a change has occurred, it is not reversible, as is the formation of a quaternary salt with the $–NH_2$ group.

This bonded phase in particular can be used to exploit differences in hydrogen-bonding capabilities between the components of a sample and this bonded phase.

7.4.1.4 Silica Gel Bonded with Ion Exchangers

Because of the ionic nature of many phytochemicals, silica gel has been modified from the early days of HPLC for use for their separation. Care has to be used with these columns since the very nature of the buffer used for the separations can dissolve the silica gel support yet leave the bonded ion exchangers intact. The result is eventual inlet-bed dissolution, forming broader and split peaks. Such dissolving can be prevented by putting a precolumn between the pump and injector filled with pure large-particle silica gel. This precolumn presaturates the mobile phase with silicate so it cannot dissolve the silica support in the analytical column [2].

TABLE 7.2
Commonly Bonded Ion-Exchange Groups on HPLC Supports

Class	Name	Structure
WAX	Aminoethyl (AE)	$-CH_2-CH_2-NH_3^+$
WAX	Diethylamine (DEA)	$-NH(CH_2-CH_3)_2^+$
WAX	Diethylaminoethyl (DEAE)	$-CH_2-CH_2-NH(CH_2-CH_3)_2^+$
WAX	Dimethylaminoethyl (DMAE)	$-CH_2-CH_2-NH(CH_3)_2^+$
WCX	Carboxymethyl (CM)	$-CH_2-COO^-$
SAX	Quaternary methyl amine (QMA)	$-N(CH_3)_3^+$
SAX	Quaternary ethyl amine (QEA)	$-N(CH_2-CH_3)_3^+$
SAX	Quaternary aminoethyl (QAE)	$-CH_2-CH_2-N(CH_2-CH_3)_2^+$
SCX	Sulfonic acid (SA)	$-SO_3^-$

A list of the most used ion exchangers are listed below in Table 7.2. Although less efficient and slower, there seems to be more use of ion exchangers bonded to a polymer. This eliminates the problem with dissolving. Since salt gradients are often used for separations on ion-exchange packings, the peaks are made narrower, and the time required for the separation of any mixture is reasonable.

7.4.2 Polymers Bonded with Hydrophilic Phases

Some of the oldest polymeric support bonded phases are the ion-exchange packings. As already mentioned, they are generally synthesized as intermediates during the initial particle-forming process, followed by reactions that convert the intermediate to an ion exchanger. Because of the difficulty of making and separating smaller-polymer particles, as well as packing them successfully, they are offered only down to an average particle size of 5 or 6 μm.

Table 7.2 lists the bonded ion-exchange packings that are most often manufactured today. Although the literature is filled with applications using ion exchangers, one unusual application for the separation of carbohydrates is the use of a cation exchanger with calcium or lead ions attached. The mobile phase is pure water, with the column operated at 75ºC–85ºC [14].

7.4.3 Cellulose

Cellulose has often been used in the separation of phytochemicals in TLC, but it has not found wide use, except as a bonding support for chiral HPLC columns. This is due to the fact that it does not have the mechanical strength to stand up to high pressures and will collapse giving high backpressures or limited flow rates.

Spherical cellulose has been bonded with many hydrophilic phases for use in chiral separations. These have become standards in the field of chiral separations. A discussion of these chiral bonded phases follows this section.

7.5 CHIRAL BONDED PHASE MODE

Many compounds with an asymmetric center, if sold as pharmaceuticals, are now required to have the isomer content analyzed. Many different chiral stationary phases are now available for use in an attempt at separating the enantiomers. The different chiral packings, often referred to as chiral stationary phases (CPSs), are discussed only briefly in the following. If it becomes necessary to begin working in this area, it is necessary to contact the manufacturers of these packings and to consult

the literature and texts to find out the details of how to use them effectively. Often they use special mobile phases or additives to generate the selectivity desired.

Of the HPLC columns available for sale today, these are the most expensive. They can cost up to four times more than a simple reversed phase column The main reason they are more expensive is that some of the phases that need to be bonded have to first be chirally separated themselves, so that they have the correct configuration for selective absorption. It is necessary that a three-point interaction between the one isomer and the bonded phase occur. Thus the isomer so attracted is delayed in moving through the HPLC column, allowing the other isomer to move faster through the column. All of the CPSs have spatial-design considerations in the bonded chiral selector to enhance this interaction.

Often the phases have both an R and an S form that can be ordered, which allow one or the other isomer of the compound of interest to elute first from the column. This could be important if one isomer is of low concentration and would be caught in the tailing end of a larger peak. By reversing the order, the smaller-isomer peak will elute before the larger peak, allowing for better quantitation.

The main classes of CPSs are (1) the brush type (so named by Pirkle), (2) cellulose ester and carbamate, (3) cyclodextrin, (4) macrocyclic glycoproteins (antibiotics), (5) protein, (6) ligand exchange, and (7) crown ethers. These are described in References [15–17].

7.5.1 Silica Gel Bonded with Chiral Phases

Silica gel is used as the support for most of the bonded CSPs shown in the preceding section. To date, the most used and available size is 5 μm for any analytical work. Most manufacturers also make a larger-particle size for scale-up work using columns with larger IDs.

For the brush type, the names of some of the CSPs are α-Burke 2, β-Gem 1, and Whelk-O1. Most bear the name of the graduate student who worked for Dr. Pickle to perfect their chemistries and to demonstrate their use for chiral separations. They have been commercialized by Regis Technologies, Inc. (Morton Grove, Il.).

For cyclodextrin and derivatives of these oligosaccharides, three cyclic forms have been bonded, alpha (six units), beta (seven units), and gamma (eight units). Enantiomers fit into the barrel shape of these molecules to different degrees, and hence separate. Versions of these CSPs are sold by Supelco/Sigma-Aldrich (Bellefonte, Penn.), under the name Cyclobond, and by E. Merck (Darmstadt, Germany), under the name Chiradex.

The macrocyclic glycoproteins are very interesting. Because of their complex structure and chiral centers, they are excellent CSPs. The research on these CSPs was done by Armstrong and his group [17]. Chirobiotic is their brand name, and they are available from Supelco/Sigma Aldrich bonded with vancomycin, teicoplanin, or ristocetin.

Proteins also have the ability to arrange themselves into structures that can promote chiral separations [18]. Three proteins have been bonded to silica for use: human serum albumin (HSA), cellobiohydrolase (CBH), and α-1 acid glycoprotein (AGP). They are sold by Supelco/Sigma Aldrich and Regis Technologies, Inc.

Ligand exchange involves bonding an organic structure containing an amine, such as L-proline, L-hydroxyproline with different spacer hydrocarbon chains. The mobile phase typically is aqueous with some amount of copper (II) acetate. This combination is used to resolve D, L amino acids and amino alcohols based on interactions forming copper complexes.

Crown ethers are rings containing several ether groups, most being oligomers of ethylene glycol. One derivatized crown ether, (+) or (–)–(18-crown-6) tetracarboxylic acid, is bonded as a CSP. It is used for the separation of amino acids and of compounds containing primary amines.

7.5.2 Cellulose Bonded with Chiral Phases

Cellulose also has a small amount of chiral selectivity because of its ordered structure [19]. This chiral selectivity can be greatly enhanced, however, by bonding it with various side groups through

a carbamate link. Amylose can be similarly bonded. These derivatives are dissolved and coated onto 5 μm or larger-spherical silica particles. There are some 20 different phases with the brand names, Chiralpak and Chiralcel, and are sold in the United States by Chiral Technologies (West Chester, Penn.). Further information, applications, and a catalog can be found on their Web site (www. chiraltech.com).

7.6 ION-EXCHANGE MODE

An alternative to separating polar species on a reversed-phase column with buffers or ion-pairing reagents is to adjust conditions in the sample solution so that the components are in their ionic form. Then the separation can be attempted using a bonded ion-exchange packing.

In using this mode, rather than attempting to change the pH of the mobile phase with a gradient, it is much easier to optimize the pH and use a change in the salt concentration to elute components. Attempting a pH change often does not work, since such a change on the surface of the packing takes much longer than the time of the completed separation. If, for instance, the pH of the mobile phase was changed from pH 4 to pH 6 during the separation, testing the pH of the eluting mobile phase after the 30-minutes gradient would show that the pH was only 4.2.

Thus most ion-exchange separations are accomplished with a set pH and a gradient of the same buffer with increasing potassium chloride concentration. KCl is used rather than NaCl since it is more soluble.

Table 7.2 shows the usual ionic groups bonded to many of the supports listed in the following.

7.6.1 Silica Gel Bonded with Ion Exchangers

Manufacturers of ion-exchange HPLC packings have used silica gel as a support for many years in spite of its tendency to dissolve under the buffer mobile-phase conditions needed for the separation. The use of a precolumn (between the pump and the injector) filled with silica gel eliminates this problem. The advantage of the silica gel support is the improved efficiency seen in the separation compared to a polymer support.

7.6.2 Cellulose Bonded with Ion Exchangers

The first support for ion exchangers was cellulose, but its use is not as prevalent today. It is a “soft” bed when packed in a column and is not stable under the higher pressures now used in HPLC.

7.6.3 Polymers Bonded with Ion Exchangers

Polymer supports have been used for many years in column chromatography and now HPLC. The polymers can be any of those listed in Section 7.3.4. They are very stable when cross-linked, and their biggest advantage is their inertness at all pHs. As already discussed, their particle size is in the 5–6 μm range. This size is sufficient to give the resolution needed since gradient elution improves the performance of larger particles.

7.7 SILICA GEL ENDCAPPING

To improve the interaction of solutes with bonded-hydrophobic-phase columns, it is desirable to bond as many of the available silanols as possible. After bonding an initial-chain-length hydrocarbon (C18 or C8) with the appropriate reagent, a second reaction can be run on that bonded silica gel. For the second bonding, a smaller-chain-length or less bulky reagent is used. This allows easier access to any remaining silanols. Such silanols can cause peaks to tail, often because of some polar interaction with amino groups on the structure of some solutes.

Endcapping is also done to prolong the life of a column should aggressive mobile phases be used. This is because initial dissolution of the silica gel backbone occurs at the silanols, not on Si-O-Si ≡ bonds (those already bonded silanol sites) [1].

As mentioned, however, often at least 50% of the silanols remain unbonded even after one or two of these second attempts to bond to them. Often, if they are truly neutral silanols (with no acidic or basic nature), they will not interfere to cause peak tailing.

Most endcapped products are so noted in the name of the product or with an *e* at the end of the name, for example, RP18e.

7.7.1 Classical Endcapping

The most commonly used endcapping reagent is a trimethylsilyl $(CH_3)_3Si–$) group. It is the smallest in size, to get into the smallest recesses of the porous silica gels. Any initial bonded C18, C8, or other phase would also prevent access to residual silanols near them. Some manufacturers claim to do double or triple endcapping reactions. This, however, does not eliminate all residual silanols, as already mentioned.

7.7.2 Polar Embedded Endcapping

Bonded reversed-phase packings like to be in a mobile phase consisting of a mixture of an organic solvent and water. In pure water, they will lose their organic solvation and collapse. This can result in an inability to do the separation after a period of time. Most often such a column can be resolvated with the methanol or acetonitrile that had been on it from the manufacturer or the user, and it will return to its original state.

To improve the stability of the bonded phases that might collapse in high-water-content mobile phases, some manufacturers have modified the endcapping to aid in keeping the phases solvated. This is accomplished by adding a polar group or groups to the endcapping reagent [9]. The actual amount of endcapping that bonds to remaining silanols is also governed by the factors mentioned in the preceding section.

7.8 PARTICLE SIZE VARIATIONS

The history of modern HPLC columns and packings began in the mid-1960s when smaller and smaller particles of silica and reversed-phase bonded silica were made and packed into columns. Mesh screens were fine for producing cuts of larger particles suitable for column chromatography but could not be used for sub-10 μm particle size ranges.

Air classification was used to produce the first generation of packings in the 15–10 μm ranges. While some manufacturers worked on these irregular silica supports, other manufacturers began to make pellicular packings. These consisted of a solid core of 30–40 μm glass coated with microfine silica and then reversed-phase bonded. These packing offered two advantages. One was their density, making them easy to pack. The second was the speed of mass transfer on and off the thin shell surface, allowing for very fast HPLC. These lost favor after the silica manufacturers successfully produced well-packed columns with 10 μm particles. It is interesting to note that the superficially porous packings have been revived again but built on a 1.7 μm solid core with a 0.5-μm-thick shell. Their brand name is Halo (Mac-Mod Analytical, Inc., Chads Ford, Penn.), and they are part of the newer sub-2 μm packing family for UHPLC.

Over the years, manufacturers of HPLC packings have offered columns with smaller and smaller particle sizes. The advantage is higher efficiency in the resulting chromatograms, for better and faster separations. An additional advantage is shown in Figure 7.4, called a van Deemter plot. It shows that with smaller particles, as the flow rate (or alternatively the linear velocity) is increased, there is less loss of efficiency with smaller-particle packed columns. This allows the chromatographer

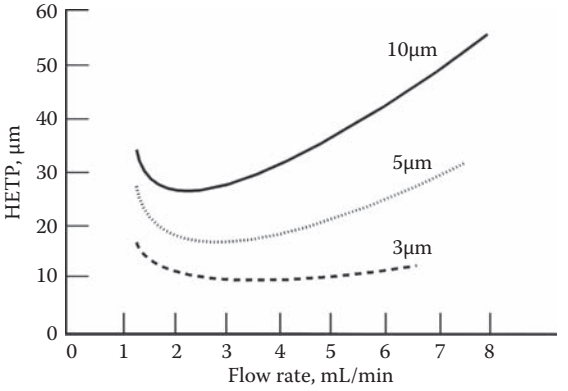

FIGURE 7.4 Van Deemter plot showing the relationship between particle size, flow rate, and height equivalent to a theoretical plate (HETP).

to increase the flow rate for a faster separation without loss of resolution. This leads to increased productivity and lower cost per analysis. In this figure, the smallest height equivalent to a theoretical plate (HETP) is the area in the curve where the highest efficiency is produced by the column.

However, the price for these advantages is an increase in backpressure. Columns made of 3 or 3.5 µm packings were the lower limit for column packings, even with shorter lengths, because of the limitations of the HPLC pump pressure capabilities (about 8000 psi) up until about 2002. Then manufacturers began to make HPLC systems with pumps and associated injectors and mixing devices that could reach 15,000 psi, so that sub-2 µm packed columns could be used successfully.

7.8.1 10 µm, 5 µm, 3 µm, and Sub-2 µm

The first challenge that column manufacturers faced at the inception of HPLC was to produce HPLC packings with a narrow particle size distribution. In the mid-1960s this was accomplished by a number of manufacturers, after a brief side trip where glass beads around 15–20 µm in size were coated with silica and then bonded with reversed phase. These gave fast HPLC since migration of the components in and out of the thin shell of active phase was so rapid.

Not only did the manufacturers have to make particles with a narrow size distribution, but they also had to come up with slurrying and packing techniques to produce well-packed HPLC columns that would be suitable and reproducible for HPLC. Often they were thick mixtures with organics, perhaps with a surfactant, which was later washed from the column before quality-control testing. In order to make more efficient HPLC columns, over time, each manufacturer began to offer columns with smaller and smaller particle sizes in shorter lengths. The reasons will become apparent in the following paragraphs.

7.8.2 Particle Size Effect on Efficiency

The smaller the particle used in any column hardware, the better the efficiency, as already shown. This allows the same separation done on a larger-size packing to be done faster with a shorter column. This reduces the cost per analysis when adding up the cost of the column, solvent, operator, and LC system.

The van Deemter plot in Figure 7.4 illustrates the change in efficiency with increasing linear velocity or flow rate. With the use of smaller particles, less loss in efficiency occurs with an increase in flow rate. This has been the driving force for manufacturers to offer the smaller-particle-size columns being used more and more today.

7.8.3 Particle Size Effect on Backpressure

The price to be paid for using columns with smaller particle size is the backpressure that is generated. Although a larger-diameter column would solve the backpressure problem, it uses too much solvent so is not practical. Even if the standard IDs of 4.6 or 4.0 mm are used, the traditional HPLC pumps that could be used at pressures up to 6000 psi were not up the backpressure challenge of 3 μm HPLC columns. They would shut down when the flow rates were increased to speed up the separation.

7.8.3.1 Ultra High Pressure HPLC Systems

About 5 years ago, a few manufacturers of HPLC systems decided to improve the pump and system (injector, mixer) to allow sub-2 μm particles to be used in HPLC columns. Thus, UHPLC systems became available. These systems allow the chromatographer to use pressures of up to 16,000 psi.

Much of the chemistry described in the preceding has become available in the sub-2 μm size range for use with these newer, faster UHPLC systems. One main point to remember is that the smaller the particle size in the column, the better a filter it is for any particulate that is not removed from the sample or mobile-phase solutions. Thus extra care should be taken in sample and mobile-phase preparation for use with these UHPLC systems.

7.9 COLUMN HARDWARE

Once the column packings are made, they are put into one solvent or another, then into a reservoir, and packed under high pressure into columns specially constructed of resistant materials. All of the components of the HPLC column—the tubing, the fittings, and the frits that hold the packing in the column—have to be made of materials resistant to the solvents and buffers they will be exposed to in their use on HPLC systems. Traditionally, most columns and their components have been made of 316 stainless steel, or, if only lower pressures are needed, a suitable plastic, like polyether-ether-ketone (PEEK), can be used.

7.9.1 Stainless Steel

Stainless steel (grade 316) is used to fabricate about 80% of the HPLC columns sold today. They vary little from one column manufacturer to another, as most buy from a few quality tubing manufacturers in their respective countries. It is important that the inside of the column be polished to a mirror-like sheen to minimize wall effects (less-well-packed areas that can cause tailing and band broadening).

The differences in column appearance from one manufacturer to another are due to the special design of the end fittings, which have been created by each manufacturer according to their specifications. They have a ferrule that locks the tubing to the fitting at each end that makes the liquid seal when the pieces are first put together. A cross-section of a capillary tube and its connection pieces and a typical HPLC column fitting is shown in Figure 7.5.

Since the connecting tubing depth (ferrule lock distance or “dimension X”) is different from one manufacturer’s columns to another’s, plastic (usually PEEK) ferrules can be used on the connecting tubing. These can then move freely on the tube, allowing self-adjustment to the correct depth so a good liquid seal is made that eliminates any dead space. Dead space would lead to band broadening (because of dilution of the sample volume) that decreases the efficiency and resolution. The plastic ferrules can be used with HPLC systems to pressures up to 4000 psi or higher depending on the combinations used, so consult the manufacturer’s Web site or contact their technical-support personnel. Search words on such Web sites are “ferrules” or “tubing sleeves.”

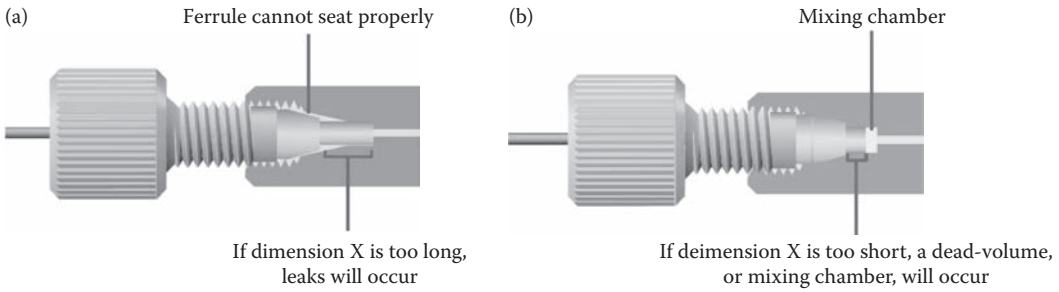

FIGURE 7.5 Cross-section of an HPLC fitting, showing the problems caused by incorrect placement of the ferrule. (Reprinted with permission from Upchurch Scientific, part of the Idex Health & Science Group, a unit of IDEX® Corporation.)

Most analytical-size columns are made symmetrically, so they can be reversed if necessary and still give the same separation. With preparative-size columns (< 10 μm ID), the exit fittings are often of a different design (to allow better collection of the sample bands as they elute from the column). Thus, the solvent flowing into the preparative column can only go in one direction (often marked on the column) when introducing any samples. All columns can be reversed when being cleaned since only pure solvents are being passed through them.

7.9.2 Polymers (PEEK, Other)

When sensitivity to any metals is to be avoided, many parts of the HPLC system and columns can be manufactured with suitable polymers. Often these need only be stable in aqueous solutions or mobile phases consisting of polar organics and water (for biopolymer separations). One need only be aware of any pressure or solvent-incompatibility problems with these columns. Otherwise, it makes no difference whether the HPLC packing is in metal or plastic hardware.

7.10 COLUMN HARDWARE, FITTINGS, AND CONNECTIONS

The chromatographer doing HPLC has a number of options when selecting the hardware in which the packing can be ordered. Often, each manufacturer has a proprietary combination of fittings and column design. The compatibility of these with the capillary connecting tube has been an issue and is discussed in the following. Two Web sites are recommended for finding out more about the fittings and connectors discussed here: *LC/GC* magazine (http://chromatographyonline.findpharma.com/) and Upchurch Scientific, one of the manufacturers of new and replacement HPLC components, including some discussed here (http://www.idex-hs.com/products/Brand.aspx?BrandID = 1).

7.10.1 Columns Complete with Fittings

The classical column hardware is a stainless steel column that is complete with the fittings necessary to connect to any HPLC system. As already mentioned, each manufacturer designs their fittings independent of other companies, so the internal measurements are different.

To make the liquid seal between the fitting and the connecting tubing, a stainless steel ferrule has to lock onto the connecting tubing and fit snugly to the coned area in the fitting (see Figure 7.5). Once the ferrule is locked onto the stainless steel connecting tubing, then it cannot be moved. This makes it usable only for this manufacturer's fitting.

The solution to this problem is to use the polymer ferrule or ferrule fitting combination previously mentioned (Section 7.9.1) which can self-adjust to any other manufacturer's fittings. These are available from most laboratory distributors.

7.10.2 Cartridge Columns

If one looks at an HPLC column, the intricate fittings are the most expensive part of the column hardware. To save column costs, manufacturers began to offer cartridge columns. These are columns without fittings that fit into a holder or have special fittings that can be used over and over again. There is no difference between a complete column with fittings and the cartridge version since the packing is the same from any manufacturer. Often, too, multiple columns are offered for even better cost savings. These should be considered for any HPLC analysis being run routinely.

7.10.3 Avoiding Dead Volume

When using HPLC, it is important to be aware of the effect of dead volume in the HPLC system. If any connecting pieces are not well fitted together and a void space exists, this void space acts like a dilution vessel. When the sample stream runs through it, the volume of the liquid in the dead space adds to the volume of the sample. This increase in sample volume gives broader peaks as each component of the sample travels through the column and into the detector. This results in less resolution and possible overlapping of peaks.

The critical areas to avoid any dead volume are between the injector and the HPLC column (A) and between the column and the detector (B). For A, the user has the option to use a small guard column (to act as a final filter for the sample). Any connections in areas A or B must be made carefully, to ensure the capillary tubing and ferrules conform to the correct locking distances to the column or fitting into which it will be placed.

When making the connection of any components for the first time, do it carefully. Assemble the pieces and hold firmly, pushing the tubing into the fitting while tightening slowly. The tightening locks the stainless steel ferrule onto the stainless steel tubing at the correct distance so the ferrule makes a liquid seal between the tubing and against the coned area in the fitting.

Remember to tighten gently and not overtighten. Any connection at the high-pressure end of an HPLC system needs to be made to be leak free at the pressures being used. Overtightening will not make the fitting connections better at giving a good liquid seal. Overtightening will, in fact, distort the ferrule's shape too much. Should something have to be changed in this part of the HPLC system later, breaking or making the connection will then be more difficult to achieve the same leak-free state.

The connection between the column and the detector also has to be free of extra dead volume. At this end of the column, however, the backpressure is atmospheric so connections need only be finger tightened to be leak free.

7.10.4 Avoiding Leaking Connections

After making and breaking connections a few times, some of the connecting pieces might have to be replaced. Each time the stainless steel ferrule is tightened into a fitting, it distorts more due to the pressure used to make the liquid seal. It is very easy to apply too much torque with a wrench. It needs to be tightened enough that there is no leakage at the pressures being used.

Some manufacturers have designed connecting pieces with gripping areas so finger tightening can be done. Often they have a larger diameter than a comparable stainless steel version. This design feature was intentional so that a sufficient amount of torque can be generated to make a leak-free seal.

It is recommended to have a number of precut, prepared stainless steel capillary tubings, ferrules, and so on in stock in the laboratory or storeroom, to be able to do quick replacement as needed. Always ensure the inner diameter of the capillary tubing is what is used or recommended for the HPLC system you are using. Consult the HPLC system manufacturer's guide for this information.

7.10.5 Use of Polymer Components

In place of the stainless steel connecting pieces, tubing, ferrules, and columns, polymer versions are available. Usually these are made of PEEK. The limitations of these in use in HPLC are, most obviously, the chemical resistance and the pressure being used. Because of their chemical limitations, always check to see that they are compatibile with the mobile phase. There is no problem using the usual reversed-phase mobile-phase components—methanol, acetonitrile, buffers, acids, or bases. If using the PEEK ferrules, they should be routinely changed once a month to prevent leakage with time and pressure.

7.11 COLUMN SIZES

Today it is not surprising to see a new HPLC packing being introduced into the marketplace, where over 30 column sizes are available to the user. Long gone is the demand for only a 4.6×250 mm HPLC column. These were fine for research and development laboratories, which needed the high efficiency generated by the longer column.

Previously, separation times averaged 15–20 minutes, or up to 2 hours if a gradient were being applied to a complex sample. Now the emphasis is on fast HPLC separation. For simple separations, most can be done in 5 minutes or, for a complex sample, in 20–30 minutes. This increases sample throughput and lowers the cost per sample analyzed. This makes the whole analysis process economical and ongoing.

The effect of the column variables is discussed in the following.

7.11.1 Column Lengths

7.11.1.1 Analytical: 300–250 mm

When using an HPLC column with an isocratic mobile phase (a mobile phase whose ratio does not change throughout the chromatographic run), then the efficiency can be calculated. That efficiency depends on the length of the column. A longer column gives the most efficiency (assuming any length is packed with the same particle size).

These columns are good for developing a separation of any mixture, be it with a few components or many. Of course, at some point in the method development, a gradient may have to be tried to elute components from a very complex mixture. When developing a method, most chromatographers will use a longer column filled with the smallest particle that is compatible with the HPLC system being used so that the backpressure does not limit the flow rates used or shut down the system. Often, columns that are 300–250 mm long are used with only 5 μm particles and can be used with standard HPLC systems that can handle pressures up to 6000 psi.

Although smaller-particle long columns could be used with the new UHPLC systems that handle backpressures up to 16,000 psi, this is not done in practice. The newer systems were designed for short, fast chromatographic runs, so only shorter columns are used. As already mentioned, the smaller the particle, the better the efficiency. The smaller particles generate higher backpressures, so compatibility with the HPLC system should be kept in mind.

7.11.1.2 Fast: 150–25 mm

Once the initial separation has been achieved on a longer column, most chromatographers see that there was more than enough efficiency for the separation. At this point, the method is optimized for speed. This means going to a shorter HPLC column, with the same packing so that the selectivity is not changed.

It is better to go to a shorter column, rather than run a longer column at a fast flow rate, to elute the sample in a shorter time. This practice will be better for the life of the LC pump and its components

and also for the other system components, since they all survive longer when the system is operated at lower backpressures.

At this point in the speed optimization, the chromatographer can change to a smaller-particle size of the same packing to get an even faster analysis time. Thus, a 4.6 × 75 mm column filled with 3.5 µm particles might be a perfect choice for the final analytical column.

7.11.1.3 Fastest: > 25 mm

These shorter columns are usually filled with 3.5 µm or sub-2 µm packings that are discussed later in this chapter. Thus, a 4.6 × 25 mm column filled with 3.5 µm particles might be a perfect choice for the final analytical column for a standard HPLC system as mentioned above. However, if UHPLC is being used, a 2.1 × 25 mm column filled with 1.7 µm particles would give the fastest separation.

Caution: At this point where the discussion turns to shorter columns with smaller diameters, it is important to consider the capabilities of the HPLC system for handling the small-volume peaks eluting from these columns. See the following discussion on bandwidths for columns of different IDs.

7.11.2 Column Inner Diameters (IDs)

7.11.2.1 Classical: 3.9, 4.0, 4.6, 10, and 22 mm

When most column manufacturers began to sell prepacked analytical HPLC columns, most used column IDs between 4.6 and 3.9 mm. These diameters are still the most sold, generally because these IDs have been written into quality-control and quality-assurance protocols. One advantage is that they give less backpressure than smaller-diameter columns. The disadvantage is that they also consume more solvent(s) at any given flow rate.

For preparative work—when larger quantities need to be separated for characterization or initial drug studies—10–22 mm ID columns are also available. Of course, their cost can be considerable. Before buying one, the user should consider the amounts needed and how long it will actually be used. It is always possible to make multiple injections on a smaller-ID column and collect fractions. This is certainly possible with any HPLC system with the automation available today.

If solvent consumption is a concern, especially with a fast HPLC separation, then a smaller-ID column can be used. See the discussion in Section 7.11.3 and Table 7.4 to compare flow rates required to give the same linear velocity and separation.

7.11.2.2 Modern: 3.0, 2.1, and 1.0 mm

As packings with smaller-particle size (with their increased efficiency and other advantages) became available, both the standard IDs already mentioned and other smaller-ID columns were made to accommodate them. As with any column of any ID, the longer the column, the more backpressure, and the smaller the particle size of the packing, the more backpressure. Table 7.3 shows a comparison of particle size, column length, efficiency, and relative backpressures.

Note that Table 7.3 shows not just the high plates per meter (N/m) but also the actual plate count in each of the shorter columns (plate count per column). For many separations, only 5000 theoretical plates are needed. In fact, more important than efficiency is the selectivity of the combination of packing and mobile phase. This, unfortunately, is also the most difficult and time-consuming step when developing a new method, if previous knowledge is not available.

The column ID also plays an important role in the sensitivity of the separation—the ability to see the components or the limits of detection. This is discussed in the following.

7.11.2.3 Special—Capillary: < 0.5 mm

Although very-small-diameter HPLC columns are available, they are used most often by researchers with very limited sample amounts, and directed to detection with various mass spectrometers. Their use by general chromatographers is limited due to the special microvolume HPLC systems

TABLE 7.3
Comparison of Particle Size, Column Length, Efficiency, and Relative Operating Pressure

Column Length (cm)	Particle Diameter (μm)	Plate Count per Column	N/m	Relative Operating Pressure
25	10	12,500	50,000	1.0
25	5	25,000	100,000	4.0
15	5	15,000	100,000	2.4
5	5	5,000	100,000	0.8
10	3	16,700	167,000	4.4
5	3	8,300	167,000	2.2
3	3	5,000	167,000	1.3
3	2	7,500	250,000	3.0

Source: Adapted from Poole, C.F., *The Essence of Chromatography*, Elsevier, New York, 404, 2003. With permission.

TABLE 7.4
Flow Rates for Equivalent Linear Velocities for HPLC Columns with Different Internal Diameters

Column Diameter (mm)	Flow Rate (mL/min)
4.6	2
2	0.38
3	0.85
10	9.5
25	59

required for their use. The bandwidths of sample components being separated are very, very small.

7.11.3 Flow Rates of Columns with Different IDs

Changing the diameter of the column in HPLC entails other considerations. In this discussion, the particle size of the packing is not changed. Whether going to a smaller- or larger-diameter column, using the same flow rate as on the initial column, let us assume it was 2 mL/min, then the flow rate in a smaller-diameter column will increase proportionally to the radius squared of the column diameter, and conversely in a larger-diameter column.

Remembering the van Deemter plot, this would result in different efficiencies being generated, so the separation could look very different or would be ruined completely, especially if the flow rate was increased in the smaller-ID columns. Essentially, the sample is now flowing through the column at a much faster rate, the sorption/desorption steps are shortened, and less resolution is obtained. To compensate for the differences, the flow rates are adjusted according to this radius-squared ratio. This is shown in Table 7.4.

By lowering the flow rate to the calculated amount, the linear velocities are made equivalent in each column. This ensures the same efficiency is generated to give the same chromatographic separation. Also, the same analysis time will result, assuming the same length of column is used for each diameter.

7.11.4 Bandwidths of Columns with Different IDs

When smaller-ID columns are used, the sample bands flowing through the column from any standard injection are smaller and higher. This is one of the possible reasons for switching to a smaller-ID HPLC column. Going to the smaller-ID column will result in increased limits of detection, which is desired in most separations where the sample size is limited.

The ratio of this peak-size increase with diameter is also related to the radius squared of the different column sizes, assuming the same volume of sample is injected. However, since the smaller-ID column contains less packing, overloading the column might become a problem. This is indicated by misshapen peaks compared to how they appeared using a larger-ID column. Thus, the sample size, either mass or volume, is typically reduced in smaller-ID columns.

7.11.4.1 Requirements for Detector Settings

Sample bands are smaller for the newer short columns with smaller-particle sizes. Likewise, if the flow rates on these are increased to give elution times in the order of 1–3 minutes, the HPLC detection system has to be able to deal with these peak widths and elution times.

Most standard HPLC systems have been made for the older, standard columns, flow rates, and separation times of perhaps 10–15 minutes. The detection system monitors the components flowing through the flow cell, and the computer records the digital output at a certain sampling rate per second, typically every 400 ms. For fast HPLC, this sampling rate has to be increased to every 50 ms to capture a more complete profile of the actual eluting peaks. Likewise, the detector has a time constant setting to improve background noise (electronic and other) by signal averaging, typically set at 2.0 seconds. For fast HPLC this is decreased to 0.05 seconds. Both of these are simple detector settings that can be changed on its detector-control settings screens. These settings can be reset (usually to the default settings) if going back to the usual slower-eluting columns.

The standard detector-cell dimensions are 10×1 mm ID, with 8 µL cell volume. This cell volume is suitable for most HPLC separations. However, whenever smaller columns and particles are used, the narrower peaks (and peak volumes) will be dispersed (broadened) within this volume. Detector cells with dimensions of 10×0.5 mm ID (one-quarter of the volume of the above-mentioned standard ones) are available and are easily installed when required. Details are given in references [21,22].

7.11.4.2 Requirements for Adjusting the Injection Volume

The adjustment of the flow rate to different diameter columns was discussed in Section 7.11.3. Consider now the adjustment of the injection volume to avoid overloading the smaller diameter column. This is done by using the equation for the volume of a cylinder. This equation is $V_{cyl} = \pi r^2 h$, where V_{cyl} = the volume of a cylinder, r = radius of the column, h = length of the column. Make sure the units are all the same, to calculate either mm^3 or cm^3.

Assuming a change is being made from a 4.6×150 mm (V_{cyl} of this column = 2.49 cm^3) to a 2.1×50 mm (V_{cyl} of this column = 0.173 cm^3) column is planned. Using a 10 µl injection on the 4.6×150 mm column, the calculation comparing the volumes of the two columns indicates that an injection volume of 0.69 µl would be required to give the same peak height when both columns were run at their appropriate flow rates.

Remember, too, that these equations work when calculating the amounts that can be loaded onto larger diameter columns if these are to be used for preparative work.

7.11.5 Performance of Columns with Different Lengths, IDs, and Particle Sizes

As the reader might have realized at this point, there are many options when choosing an HPLC column, not only the packing. Once the separating conditions are achieved, then optimization for

speed and efficiency is considered. The purpose of this final optimization is to minimize sample analysis times as much as possible. At the same time, solvent consumption (hence, cost) is also reduced.

7.11.5.1 Effect of Column Size on Efficiency and Resolution

The efficiency of a separation depends on the particle size of the packing and the length of the column only. No matter what the ID of the column, as already discussed, with any given column length, with the adjustment of the flow rate to give the same linear velocity, the separation will be equivalent in resolution and time.

7.11.5.2 Effect of Column Size and Particle Size on Sensitivity

Besides the separation of components in a mixture, the sensitivity (or limits of detection) is equally important. Just as the particle size plays a part in the efficiency of the separation, the column dimensions and packing size can be chosen to improve the sensitivity. This certainly brings a large increase in detectability for any separation, yet another reason for using the newer packings in the smallest-possible column dimensions that match up with the HPLC equipment available in the laboratory.

7.12 COLUMN CARE FOR LONGER LIFE

Today HPLC columns can be relatively expensive, perhaps in the $400–600 range, much more if using chiral columns. As such, the more samples that can be put through it and the longer it can be kept from having problems, the more reasonable the expense of the initial purchase, with respect to cost per sample. This section will cover some important considerations for preventing components from getting into the column that would degrade its performance or collect, causing backpressures to increase to the point where the HPLC pumping system would shut down. In addition, some column cleaning points will be mentioned. As long as only soluble impurities collect at the inlet of a column, they can be dissolved from the column with certain solvent systems.

The first general rule regarding solvents and samples that go into an HPLC column is that all components should be *miscible* and *soluble* in one another. Thus the sample must dissolve completely in the mobile phase and in any subsequent mobile phases should a gradient be used. Likewise, do not inject a sample into a column if the sample solvent is not miscible with the solvent now in the column. Not heeding this advice will result in multiple problems for both the column and sample, so that no meaningful separation can be accomplished.

For instance, heptane is in most bonded amino columns when they come from the manufacturer. However, if using this column for a carbohydrate separation it will need to be changed over to a mixture of acetonitrile and water, perhaps 80:20. It is necessary to use a solvent of intermediate polarity, like chloroform, to first wash the column (only about 20 column volumes; see Table 7.5), then to the acetonitrile–water mobile phase to be used for the separation.

7.12.1 Standards and Pure Components

Always use the purest standards and components possible for the mobile phase. Although many catalogs indicate the purity of a standard, it has to be tested to see if it meets one's own purity standards and contains no impurities that would compromise the analysis being performed. It might be necessary to clean up the standard by column chromatography ("flash" chromatography), TLC, or HPLC to make the pure standard needed. The supplier of the standard might be able to help with a suitable cleanup method. When buying a standard, look to see if it is available from more than one supplier. After checking their specifications, perhaps the highest-purity one can be found.

TABLE 7.5
Column Volumes Required for Column Cleaning

Column Dimensions (mm)	Column Dead Volume (mL)	20 × Col Dead Volume (mL)
4.6 × 25	0.061	1.2
4.6 × 50	0.122	2.4
4.6 × 100	0.243	4.9
4.6 × 150	0.365	7.3
4.6 × 250	0.608	12.2
3.0 × 25	0.017	0.3
3.0 × 50	0.034	0.7
3.0 × 100	0.068	1.4
3.0 × 150	0.101	2.0
2.1 × 25	0.006	0.1
2.1 × 50	0.012	0.2
2.1 × 100	0.023	0.5
10 × 100	3	50
10 × 250	6	125
25 × 100	39	781
25 × 250	98	1953

Calculated as π [radius(cm)]^2 × height (cm) = volume of cylinder; dead volume ≈ half the volume of cylinder.

7.12.2 Biological Samples

Since all the components in plants are of concern in this book, good sample cleanup is essential. Most papers on the subject of their separation also detail the cleanup method used. This can always be improved on to limit problems with the separation.

Perhaps the separation is not reproducible, is losing efficiency, and is developing high backpressure after only a few dozen separations. Most likely this indicates that impurities are collecting on the column. Perhaps the reference being followed used a 5 μm column, and it is being changed to a sub-2 μm column. The separation will work, but the filtration for a 5 μm column is not as rigorous as that needed for a sub-2 μm column. This example points out the need at times for going back to the sample-preparation step to rework it or add an additional cleanup step.

7.12.2.1 Sample Preparation

There are many sample-preparation steps that can be used depending on the source of the sample and the cleanup necessary to get reproducible analytical results. They include cell disruption, dialysis, ultrafiltration, lyophilization, reconstitution, Soxhlet extraction, homogenization, precipitation, mixing, digestion, grinding, derivatization, votrexing, drying, liquid–liquid extraction, solid-phase extraction (SPE), concentration, centrifugation, sonication, evaporation, dilution, pH adjustment, weighing, addition of internal standard, and perhaps a dozen or more others relating to specific samples.

In many studies of HPLC work, sample preparation is the most time-consuming step, even if automated. Still, it is necessary in order to get reproducible results. Time spent on sample-preparation studies will pay off in the future, since many problems that can arise with an HPLC protocol are related to failure to remove impurities or interfering components.

Only a few of the possible sample-preparation methods are briefly discussed here. Most papers cited in this text have a sample-preparation section that will be informative. Texts on the subject can be consulted for further information [23,24].

7.12.2.1.1 Solvent Extraction

In spite of the availability of SPE (see next section), almost 50% of the papers being published continue to use solvent extraction (or liquid–liquid extraction) as the method for isolating components from plants. The reason for this is likely the limited time to move on to a separation, so solvent extraction is used by default. The only disadvantage is the use of relatively large volumes of solvent and its subsequent disposal.

There are disposable columns filled with diatomaceous earth that can be used for liquid–liquid extraction. The aqueous sample is added to these columns after the sample solution is adjusted for pH, perhaps with the addition of salt (for salting out). After the sample has been placed in the *dry* column (they are not preconditioned with solvent as are SPE columns), an organic solvent is passed through it to elute a desired fraction.

Unlike the SPE columns discussed in the next section, the column size used has to be in proportion to the amount of sample to be placed in it. These columns are sold to hold 1 mL, 5 mL, 10 mL, and so on of a sample. Any volume outside of the stated volume of these devices will run through, but no enrichment of the components occurs. The diatomaceous earth is only acting as a sponge to hold the sample volume for subsequent fractionation of the sample with an organic solvent(s). They are known by the trade names Extrelut NT (Merck KGaA, Darmstadt, Germany) or Chem Elut (Varian, Inc., Palo Alto, Calif.). Their advantages are their ease of use, use of only a small amount of eluting solvent(s), and no cleanup of glassware. Contacting these companies or searching the Internet with the trade names as search words can be a source of reference papers or protocols using these devices.

7.12.2.1.2 Solid-Phase Extraction (SPE)

SPE is just a miniversion of column chromatography that has been used for the past 40 years to prepare samples for any number of analytical techniques. Various manufacturers make these minicolumns filled with silica, bonded silica, or polymers. There are bonded reversed phases, ion exchangers, and mixed-mode versions. Since the use of SPE is so well established, it is only a matter of searching the literature or the manufacturers' Web sites to find many detailed sample-cleanup methods.

When using these devices, most have to be preconditioned, that is, prewetted with some solvent to solvate the surface or bonded phase to allow it to absorb the components from the initial solution. Thus, most bonded C18 SPE tubes are first washed with methanol or acetonitrile.

Since the sorbents in an SPE column are active absorbers (whether bonded or a simple silica gel), the selected components in the sample solution are absorbed to the surface. Thus, it is possible to pass 1–100 mL or more of a dilute sample solution though a SPE C18 column containing only 100 mg of bonded phase. There is an upper limit, however, so experiments must be done to determine the volume and/or mass that any SPE column can hold without overloading it to the point where it no longer collects components from the sample.

Elution of the SPE column can then be done in stages with stronger eluting solvent(s) (i.e., fractionation) into more easily separated samples. Since extraction of plant materials will give thousands of possible components, fractionation into less complex mixtures is necessary.

The time-consuming steps are determining the sample loading and elution conditions that will give the best results. The experimental work can be made easier by using TLC, since many fractions from many different experiments can be spotted, developed, and visualized if necessary, to see the results. The companion volume of this book will be a source of basic information giving the necessary details to get started [25].

Once the correct phase and conditions are determined for sample preparation with SPE columns, it is possible to do the same procedure in 96 well plates to decrease sample-preparation time. It is recommended that, once a specific brand is chosen, the user purchases at least one other lot of the product from the manufacturer to ensure reproducibility, just as was recommended when using an HPLC column.

To find the various manufacturers of SPE columns, sorbents, or cartridges, search the internet, using, "solid phase extraction sorbents" or similar in the search engine.

7.12.2.1.3 Online Sample Preparation

It is possible to prepare the sample for injection by using a multiport switching valve (an eight-port valve rather than the usual six-port valve). In the one position is a minicolumn similar to a SPE column. The sample is collected in it, perhaps washed with a cleaning solvent, then back-flushed into the analytical HPLC column for separation.

Keywords for searching for such methods include "column switching," "RAM" (restricted access media), "ADS" (alkyl diol silane), and "ISRP" (internal surface reversed phase). The latter are special sample-preparation materials with inner pores designed to collect solutes or their metabolites and an outer surface designed to repel proteinaceous material from a biological sample and send it to the waste stream.

7.12.3 Mobile-Phase Components

Doing suitable sample preparation of the plant extracts has been discussed in the preceding sections. Perhaps 1000 samples will be put into an analytical column before noticeable changes in the chromatogram will be seen, and cleaning of the column may be needed. If 10 μg/10μL is injected each time, this amounts to 10 mg/10mL of components and sample solvent that have been injected into the column. This would certainly be considered a very small amount of either by any chromatographer.

Continuing with these 1000 analyses, at 1 mL/min, with a 10-minutes separation time, the amount of mobile phase flowing through the column would be 10 L. Certainly this would be considered a rather large amount of solvent. As such, it is reasonable to acknowledge that the mobile phase and any components also have to be as clean as possible to avoid contributing to the collection of impurities in the analytical column. General rules will be discussed in this section.

7.12.3.1 Organic Solvents

Only a few years after analytical laboratories began to use HPLC, solvent manufacturers became aware of the market potential for solvents made to suit the needs of liquid chromatographers. Now there are many manufacturers of HPLC-grade solvents.

The general specifications are that they be filtered to eliminate particulate matter, so all are filtered with 0.2 μm membrane filters. They have to be manufactured to purity to have low UV absorbance if they have transparency in this region, particularly for acetonitrile, which can be used to 200 nm. Often UV spectra are available for specific lot number on the manufacturer's Web site.

When using any manufacturer's solvents, always keep a record of the lot numbers in case problems arise. Most solvents the chromatographer uses are pure, but certain solvents like chloroform and tetrahydrofuran are available with and without preservatives, since they can break down with time. If these or other solvents that contain preservatives are used in HPLC work, the preservatives must not interfere with the separation or change the selectivity in any way. If doing scale-up work using a larger column, do not use a lesser-grade solvent to save money since it might introduce impurities into the collected fractions.

Much more can be learned about HPLC solvents and their use by consulting Reference [26].

7.12.3.2 Water

Since the majority of separations of phytochemicals use bonded reversed-phase columns, mobile phases composed of water and a polar organic solvent are going to be found most often. Thus HPLC-grade water is necessary to make up the mobile phases. It can be purchased from the manufacturers of HPLC solvents, but most laboratories use water-purification systems that produce type 1 water (the highest-grade water, with the lowest resistivity) on demand.

Most contain cartridges with ion exchangers and carbon. The more expensive models have systems that automatically recharge the ion exchangers; other models are monitored and cartridges have to be replaced periodically. All systems have monitors showing the resistivity of the water being produced. These water-purification systems are already found in most laboratories. They are available in a number of configurations to produce the quantity needed, from a few liters to 50+ liters per day. These systems are available from the manufacturers and/or many laboratory distributors.

7.12.3.3 Buffer Salts

Most mobile phases used for reversed phase chromatography include a buffer to control the pH. Controlling the pH of the components gives better separation, peak shape, and reproducibility. The pH adjustment affects the components in the sample that can ionize. Reversed-phase separations work best with associated species, that is, RCOOH, that are easily absorbed onto the bonded C18 or C8 phase. The ionized species, that is, ROO^-, is not absorbed but will elute near the beginning of the chromatogram. Or if the exchange between the two is rapid enough, then a broad peak for the organic acid is seen. The same is true of amino, NH_3 / NH_4^+-containing compounds; the free base separates better on a reversed-phase column.

HPLC-grade buffers are available from laboratory chemical manufacturers and suppliers today and should be used. They are made to have low particulates and low UV absorption, as are the HPLC-grade solvents.

Even when using HPLC-grade reagents in the mobile phase, once mixed, they should still be filtered with a 0.2 µm membrane filter before use in the separation.

7.12.3.4 Acids and Bases

In a few instances, simply adding an acid or a base will suppress ionization in a reversed-phase separation, and a buffer is not needed. A relatively small amount of either (0.1–0.5% by volume) is needed to accomplish this control. Weak bases are used (diethylamine or triethylamine) to prevent any attack on the silica support for a method that will be used over the long term. Acids such as acetic, formic, phosphoric, and perchloric acids have all been used without deleterious effects on the long-term stability of the HPLC column. These acids and bases are also now available from manufacturers and their distributors as HPLC grades.

7.12.4 Filtration of the Mobile Phase and Samples

As already mentioned, even if using only HPLC-grade reagents for the mobile phase, filtration before use is always recommended. The smaller the particle size in the HPLC column, the finer the final filter should be. The most used membrane filter is 0.5 µm for 3–5 µm packed columns. It is recommended that 0.2 µm membrane filters be used for all the newer sub-2 µm HPLC packings. Always consider the compatibility of the membrane filter with the mobile phase so that no dissolving of the membrane occurs. Membrane manufacturers' Web sites always contain a compatibility chart that can be consulted.

The volume of the mobile phase that can be filtered before the membrane clogs, slowing the filtration, can be increased by using a glass-fiber prefilter in the membrane-filter holder. The size of the prefilter is chosen so that it just lies on the membrane. The prefilter will collect particles

throughout its depth, preventing the particles from clogging the pores of the membrane filter. If the filtration rate slows down, the prefilter can be replaced with a new one, and more mobile phase can be filtered.

7.12.5 Apparent pH of Solvent Mixtures

When a buffer solution is added to a polar organic solvent, the environment around the solvated ions and protons changes. Since the protons are now solvated with the combination of water and organic solvent, the pH changes. The pH (defined in water only) is now referred to as the apparent pH or pH* [27].

In most combinations of buffer and organic solvents, the apparent pH increases. The increase is related to the buffer concentration and the ratio of organic solvent to water. Thus, the apparent pH is often 0.2–0.5 higher than when measured with the buffer solution only.

It is important to correctly state (or translate if in a method) how a buffered mobile phase is made up. The following sentences are correctly stated: The mobile phase was methanol–0.1 M KH_2PO_4, pH 4.2, adjusted with 1 M H_3PO_4 (80:20). The apparent pH of the mobile phase was 4.8.

To have all the mobile-phase information, the pH of the buffer is adjusted first, and then it is mixed with the organic solvent. The pH* can then be measured with the usual pH electrode, which gives a close approximation of the apparent pH. After measuring the apparent pH, the pH electrode should be put in hot water to dissolve out any KCl that might have precipitated out in the capillary bore. The saturated KCl in the pH electrode would have formed crystals in its capillary bore when placed in the mobile phase comprised of organic solvent and buffer because it would not have been soluble. No harm is done to the pH electrode with such a measurement and hot water wash.

7.12.6 Use of Guard Columns

When doing an HPLC separation with plant extracts, even if a sample-preparation technique is used, there may still be impurities in the sample that would collect on the analytical LC column. Some of these will collect and can be washed off the column with another mobile phase or wash solvent. Others, however, may be irreversibly bound. This then would lead to column deterioration, and poorer separation and high backpressure.

When doing these separations, it is always a good idea to incorporate a guard column ahead of the analytical column to keep the analytical column from collecting these impurities. The guard column is placed between the injector and the analytical column. It is about 1/20 the size of the analytical column and is most often filled with the same packing as found in the analytical column. The guard column might have its own holder or might be a part of the cartridge column system. This hardware varies from manufacturer to manufacturer.

The guard column is changed when the backpressure increases to 500–800 psi above the initial backpressure of the column. Do not try to clean a guard column; just replace it with a new one.

7.12.7 Use of Precolumns

As already mentioned, the major drawback of silica gel is that it will dissolve if the ionic strength of a buffered mobile phase is high (greater than 0.1 M) and if the pH is higher than 11. Even if bonded, with time the residual silanols will be the sites of dissolution. The alternative of using another support such as alumina, zirconia, or a polymer has been mentioned.

It is possible to use a silica supported bonded phase under the conditions previously described above if a precolumn filled with large-particle silica gel (40–63 μm) is used. This precolumn is placed between the pump and the injector and is the same size column as the analytical (or preparative) column. As the mobile phase passes through the precolumn, the mobile phase will slowly

dissolve the silica; silicate ion is thus put into the mobile phase. When the mobile phase enters the analytical column, it is already saturated with silicate and cannot dissolve more from the much more expensive analytical column [2].

Silicate in the mobile phase is a fact that cannot be avoided if using the mobile phases described. Certainly it is better to have this happen with the use of a precolumn. If fractions are collected, simply drying them in a glass test tube or container, then redissolving them in an organic solvent will leave behind the silicate on the glass surface.

In the literature, some chromatographers, depending on their background, use the terms *guard column* and *precolumn* interchangeably. Thus, one must read further into the article to determine which they are using. The HPLC terms as used in this chapter are those described in the ASTM standard practice [28].

7.12.8 Column Cleaning and Regeneration

Once an HPLC column is not able to meet its system suitability test, meaning it is not performing to the level it should be with respect to resolution of a critical pair of peaks, or the peaks are broadened or split, then three courses of action can be taken.

The first option is to reverse the analytical column. This places the clean bottom at the top to become the inlet. Subsequent samples are introduced into a relatively clean and undisturbed bed. It is the integrity of the inlet bed that determines the placement of the sample bands that then are carried through the column for the separation. As already mentioned, if using a preparative column (with an ID > 8 mm), the reversing of the column will not work since these columns have different inlet and outlet fittings.

The second option is to replace the column, allowing enough time for equilibration. Some new columns take longer to equilibrate than others. Reversed-phase columns generally equilibrate in 15–30 minutes. If the mobile phase contains an ion-pairing reagent or is a buffer used to equilibrate an ion-exchange column, it is best to equilibrate overnight. The equilibration is complete when the baseline is stable and the retention times of the components of the standard and sample are not changing with time.

The third option is to clean the column of any impurities that might have collected at the inlet. First, if a guard column was used, and its replacement did not improve the separation problems, most likely more impurities were collected than the guard column could hold, so the additional impurities were simply carried into the analytical column, where they collected.

Begin by throwing away the guard column, then remove the analytical column from the LC system and reverse the flow (that is, reverse the column). Since the column is being washed with solvent, it need not even be attached to an LC system but only to a pump, perhaps in a laboratory hood, to do the cleaning. Set up the LC system to do another analysis while the older column is being cleaned.

Cleaning a column requires a minimum of 20 column volumes of each solvent in a series. A column volume is approximately half the volume of the empty column (or the volume of a cylinder). These volumes are shown in Table 7.5.

Depending on the support and bonding chemistry, different series of cleaning solvents are used. The recommended series summarized in Table 7.6 are for HPLC packings based on a silica gel support (irregular, spherical, monolithic, Type A or Type B, or silica hybrids), since these are used most by analytical chromatographers. The reader using other HPLC supports should obtain recommendations from their manufacturer.

Each solvent in the table either is used to clean a specific portion of the support or bonded phase or is an intermediate solvent to prevent immiscibility or insolubility with the subsequent solvent. Drying of the solvents is done by placing some oven-activated spherical 4A molecular sieves into a small bottle and adding the few milliliters of the solvent needed, and letting it stand overnight to remove any water. More details of column care can be found in References [2,29,30].

TABLE 7.6
Cleaning Solvent Series for Silica-Based HPLC Packings

Silica Gel or Polar Bonded Phases (NH2, Diol, Cyano)	Reversed-Phase Bonded (Phenyl, C2, C8, C18, C30)	Bonded Ion Exchangers (WAX, SAX, WCX, SCX)
Down the series to clean	Down the series to clean	Down the series to clean
Heptane	Water	Water
Chloroform	Methanol	0.1 M Buffer
Ethyl acetate	Chloroform	Water
Acetone	Methanol	0.01 M Sulfuric acid
Ethanol	Water	Water
Water	0.01 M Sulfuric acid	Acetone
	Water	Water
Up the series to activate completely or to the solvent polarity to be used		0.01 M Disodium EDTA
	Then with the mobile phase to be used	Water
(All solvents except water dried with 4A spherical molecular sieves)		
		Then with the mobile phase to be used

Source: For details, see Rabel, F., *J. Chromatogr. Sci*., 18, 394–408, 1980. With permission.

7.12.9 Column Storage

When a column is not being used, even if connected to the LC system, it should only have the mobile-phase solvents in it, minus any acids, bases, or salts. Thus, if a mobile phase comprised of acetonitrile–0.2 M KH_2PO_4, pH 4 (80:20), had been used for the separation, before shutting down the system, it should be washed with about 10 column volumes of a mixture of acetonitrile–water (80:20).

This wash removes any salts from the entire system to prevent attack on the column packing and prevents any corrosion in the LC system should there be a mismatch in the stainless steel components used in its construction. It will also prevent any salts from precipitating around the pump or injection components and so prevent scratching of their liquid seals, which can cause leaks to occur.

If storing the column for use at another time, it is recommended that the column be washed as described to remove as many impurities as possible. It is then stored in the mobile phase minus that acid, base, or salts, as already described.

It is always safe to store a column in the solvent in which it was supplied when purchased, but it is more convenient to store it in the solvent system in which it is to be used. Place a note or tag on the column to indicate the storage solvent so no immiscibility problems are encountered the next time it is used.

It is recommended that a "column" laboratory notebook be used for recording the history of the columns used in the laboratory. This can include the catalog number, lot number (from the test chromatogram, which should also be archived), original use information (including some separation of standards information and backpressure), and its history of use (what mobile phase was used, how many samples were applied during each periodic use, and, of course, who used it if others in the laboratory might be sharing columns or the responsibility of testing the next batch of samples on any column).

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8 Separation of Nonionic Analytes: Reversed- and Normal-Phase HPLC

Mirosław Hawrył and Wojciech Markowski

CONTENTS

8.1 INTRODUCTION

In the last 15 years liquid chromatography (HPLC), connected with mass spectrometry (MS) and nuclear magnetic resonance (NMR), became the basic instrument in the analysis of natural products, in which the main aim is, first of all, isolation, identification, and in many cases quantitative analysis of their components.

High performance liquid chromatography (HPLC) is one of most popular methods for the analysis of medicinal plants. It is an easy method to learn and use, and it is not limited by the sample compound's volatility or stability. Generally, HPLC can be used to analyze almost all the compounds in herbal medicines. Thus, over the past decades, HPLC has been the most often applied method for analyzing natural plant materials. Reversed-phase columns are probably the most popular columns used in the analytical separation of herbal medicines. It is necessary to notice that the optimal separation conditions for HPLC involve many factors, such as the different compositions of the mobile phases, their pH adjustment, pump pressures, and so on. Thus, a good experimental design for the optimal separation seems in general necessary [1,2]. In order to obtain better separation, some new techniques have recently been developed in the field of HPLC research. These are micellar electrokinetic capillary chromatography (MECC) [3], high-speed countercurrent chromatography (HSCCC), low-pressure size-exclusion chromatography (SEC) [4], reversed-phase ion-pairing HPLC (RP-IPC-HPLC) [5,6], and strong anion-exchange HPLC (SAX-HPLC) [7]. They provide new opportunities for good separation for some specific extracts of some herbal medicines. At the same time, the advantages of HPLC lie in its versatility for the analysis of the chemical compounds in herbal medicines. However, the commonly used detector in HPLC, namely, a single-wavelength ultraviolet (UV) detector, seems to be unable to fulfill the task, since lots of chemical compounds in herbal medicines are nonchromophoric. Consequently, a marked increase in the use of HPLC

TABLE 8.1
Types of Samples Analysed by Use of HPLC Methods

Regular Samples	Neutral and Ionic	Isocratic
		Gradient
		Ion-pair
		Nonaqueous reversed-phase HPLC
		Normal phase
		Inorganic ions
		Isomers
		Enantiomers
Special Samples	Biological samples	Peptides
		Carbohydrates
		Nucleotides
		Proteins
		Nucleic acids
		Carbohydrates
	Macromolecules	Synthetic polymers

analysis coupled with evaporative light scattering detection (ELSD) in a recent decades demonstrated that ELSD is an excellent detection method for the analysis of nonchromophoric compounds [8–10]. This new detector provides the possibility of direct HPLC analysis of many pharmacologically active components in herbal medicines, since the response of ELSD depends only on the size, shape, and number of eluate particles rather than the analysis structure and/or chromophore of analytes as the UV detector does. In particular, this technique is quite suitable for the construction of fingerprints of plant medicines. Moreover, the qualitative analysis or structure elucidation of the chemical components in plant medicines is not possible using simple HPLC but instead requires the application of hyphenated HPLC techniques, such as HPLC-MS and HPLC-NMR [11].

Samples analyzed by HPLC can be divided into regular samples and special samples (see Table 8.1). Regular samples are defined as typical mixtures of small molecules (<2000 Da) that can be separated using more-or-less standardized starting conditions. Exceptions or special samples are usually better separated with a different column or customized conditions.

Regular samples can be further classified as neutral or ionic. Samples classified as ionic include acids, bases, amphoteric compounds, and organic salts (ionized strong basics or acids). If the sample is neutral, buffers or additives are generally not required in the mobile phase [12].

Phytochemicals are the individual chemicals from which plants are composed. The active components occurring in plants fall into many chemical groups: lipids and derivatives (hydrocarbons, functionalized hydrocarbons, terpenes), aromatics (tetrapyroles, phenols), carbohydrates, amines and alkaloids, amino acids, nonprotein amino acids and proteins, nucleic acids, nucleotides, and nucleosides [13].

8.2 METHOD DEVELOPMENT FOR NONIONIC ANALYTES

Reversed-phase chromatography (RPC) is the first choice for most regular samples. It is typically more convenient and rugged than other forms of HPLC and is more likely to result in a satisfactory final separation [14,15]. Reversed-phase columns are efficient and stable. The results obtained using columns are reproducible. Detection is often easier in RPC because of the solvent used.

Although many organic compounds have limited solubility in the aqueous mobile phase, this is not a practical limitation because only small amounts of sample are usually injected. When sample solubility in water-containing mobile phases is poor, normal-phase chromatography (NPC) is preferred instead. Similarly, samples that are unstable in aqueous media can also be separated by NPC using nonaqueous solvents.

Nonaqueous reversed-phase HPLC (NARP) is reserved for very hydrophobic samples that are retained strongly or not eluted with 100% acetonitrile as the mobile phase (e.g., lipids). The mobile phase for NARP separations will be a mixture of more polar (A) and less polar (B) organic solvents. Often the A solvent will be acetonitrile or methanol, while B solvent can be tetrahydrofuran, chloroform, methylene chloride, acetone, methyl-*t*-butyl ether, or various mixtures of these solvents.

8.3 REVERSED-PHASE HPLC

The most popular system, used for over 50% of separations, is reversed-phase HPLC on octadecyl-bonded silica gel. Analytes are retained on the stationary-phase materials based on their hydrophobicity. This means that polar compounds are eluted faster than nonpolar compounds. The higher the hydrophobicity of the stationary-phase surface, the longer the retention times of the analytes will be. Bonding of donor-electron groups onto the stationary-phase surface results in a stronger retention of compounds containing dipoles.

In some cases, interactions between untreated silanol groups on the stationary phase and hydroxy groups on the analyte increase the selectivity; however, polar groups on the stationary phase are generally eliminated to obtain inert stationary-phase materials, which are more stable in long-term operation.

Three organic solvents, acetonitrile, methanol, and tetrahydrofuran, are usually used as the organic modifiers. Increasing the concentration of the organic modifier decreases the overall retention times, but changes in relative retention times depend on the properties of the analytes.

The general relationship between the type of solute and its retention can be seen by comparing the retention factors, k, of a set of standard compounds with their octanol–water partition coefficients, that is, the log P value, as a measure of their relative solubility in water. The logarithm of the retention factor, log k, of these compounds measured in 50% aqueous acetonitrile on an octadecyl-bonded silica gel column shows a close linear relationship.

However, the elution behavior of aromatic and aliphatic compounds is often different (even though their carbon numbers and van der Waals volumes are very similar) in eluents containing different organic modifiers. These variations are due to differences in the solubility of analytes in the organic solvent.

Five organic solvents (acetonitrile, methanol, tetrahydrofuran, acetone, and dimethylformamide), which are homogeneously miscible with water, have been used as modifiers to study the relationship of the solvent's selectivity to the analytes' molecular properties (see Figure 8.1).

8.3.1 Isocratic

In isocratic elution, the mobile-phase composition is constant during the whole chromatographic process. Isocratic separation works well for many samples, and it represents the simplest and most convenient form of HPLC. For some samples, however, no single mobile-phase composition can provide a generally satisfactory separation.

8.3.2 Gradient Elution

The programming of solvent composition (gradient elution) is a very useful method for complex samples, especially from medicinal-plant extracts. For details, see Chapter 10.

Advantages of gradient analysis are [16]:

- Better suitability for complex samples and applications that require quantitation of all peaks or multiple analytes of diverse polarities
- Better resolution of early- and late-eluting peaks
- Better sensitivity of late-eluting peaks
- Higher-peak capacity (fitting more peaks on the chromatogram)

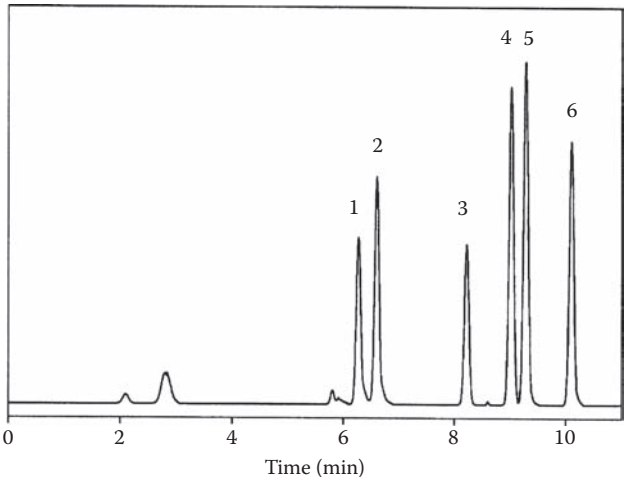

FIGURE 8.1 Separation of polycyclic aromatic hydrocarbons. Conditions: coulumn, 15 cm × 4.6 mm, i. d.; stationary phase Vydac TP C18, 5 μm; mobile phase 1.5 ml min^{-1}; water–acetonitrile, linear gradient from 50 to 100% acetonitrile between 3 and 10 min; UV detector, 254 nm (From Meyer, V. R., *Practical High-Performance Liquid Chromatography*., J. Wiley&Sons, Chichester, New York, Weinheim, Brisbane, Singapore, Toronto, 2004. With permission.)

Disadvantages of gradient elution are:

- Requirement of more complex HPLC instrumentation
- More difficult method development, implementation, and transfer
- Typically, longer assay times, since column must be equilibrated with the initial mobile phase

Several additional parameters need to be optimized in gradient analysis [12,17] that are not needed in isocratic HPLC. These are initial and final mobile-phase composition, gradient time or duration, flow, and sometimes gradient curvature (linear, concave, or convex). Optimization of all these parameters is not intuitive but can often be readily accomplished by software simulation programs [16].

8.3.3 Optimization

The first step in RPC is usually using a solvent gradient from 5 (or 10%) to 100% solvent B. There are some suggestions for nonionic samples are given by Snyder et al. [12,17].

1. Use a C_8 or C_{18} stationary phase with unbuffered water–acetonitrile, at ca. 40°C if temperature adjustment is possible, otherwise at ambient temperature.
2. Adjust the percentage of solvent B or the gradient range for retention factors between 1 and 10 (or 1–20 for difficult separations). If the separation is inadequate, adjust the selectivity in the following order:
3. Change the organic B solvent.
4. Use a mixture of organic B solvents.
5. Change the stationary phase (preferably to a type that has markedly different properties). It is probably necessary to start at step 1 again.
6. Change the temperature.
7. Optimize the physical parameters such as column dimensions, particle size, or flow rate [18].

If the separation is still poor after all these proposals have been tried, it is necessary to use another method (normal-phase, ion-exchange, adsorption, or SEC).

Some selected applications are described in literature [19–34]. For others, see the relevant chapters in this book.

8.4 NORMAL-PHASE HPLC

Normal-phase HPLC was formerly called adsorption HPLC. Simple or mixed organic solvents are used as the eluent, and adsorbent as the stationary-phase is more polar than the eluent. Hydrogen bonding is one of the most important molecular interactions between sample molecules and the adsorbent. When no molecular interaction is recognized, such chromatography is called size-exclusion HPLC (see Figures 8.2 and 8.3).

The basic molecular interactions in normal-phase HPLC are electrostatic forces. The sample molecules are retained strongly by hydrogen bonding when the sample molecules themselves or the adsorbent act as both a hydrogen-bond acceptor and donor. The specificity of normal-phase HPLC is the result of the direct formation of a strong molecular interaction between sample molecules and the adsorbent. The position of substitution of sample molecules directly affects the separation of isomers. The substituent effect is weak in reversed-phase HPLC. Therefore, normal-phase HPLC is suitable for the separation of isomers, such as *cis-trans*, *ortho-meta-para*, and steric isomers. The steric effect is especially important for chiral separations on suitable chiral columns.

Normal-phase HPLC is thus a steric-selective separation method. The molecular properties of steric isomers are not easily obtained, and the molecular properties of optical isomers estimated by computational chemical calculation are also not easily obtained. Therefore, the development of methods to predict retention times in normal-phase HPLC is difficult compared with reversed-phase

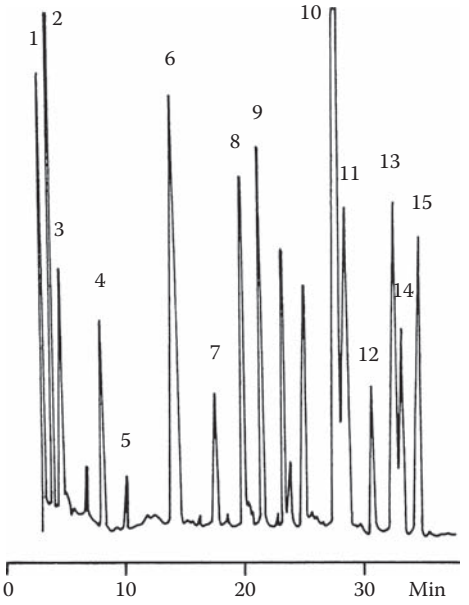

FIGURE 8.2 Separation of carotenoids from red pepper. Conditions: sample, saponificated extract from red pepper fruit; column, 25 cm × 4.6 mm i. d.; stationary phase, Spherisorb 5 μm; mobile phase, 1 ml min^{-1} petroleum ether–acetone, linear gradient from 5 to 25% acetone in 30 min; visible-range detector 460 nm. (From Almela, L., Lopez-Roca, J.-M., Candela, M. E., and Alcazar, M. D., *J. Chromatogr. A*, 502, 95–106, 1990. With permission.)

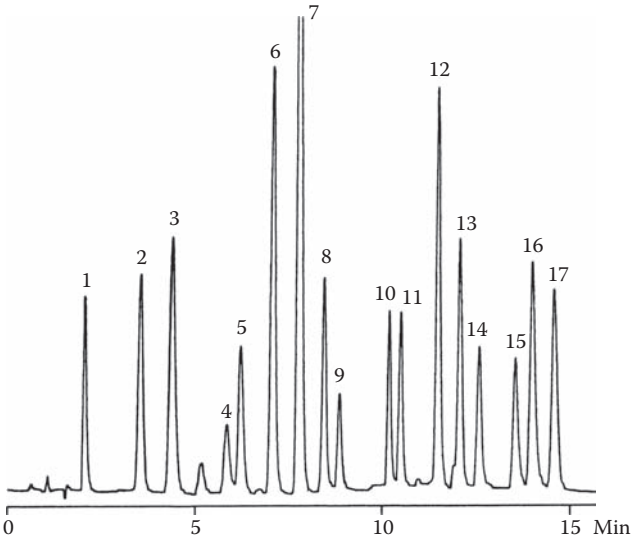

FIGURE 8.3 Gradient separation on silica. Column 25 cm × 3.2 mm; stationary phase: LiChrosorb SI 60, 5 µm; eluent A: n-hexane; eluent B: dichloromethane; gradient: 0–2 min 100%A, 2–12 min 0–80%B, flow 1 ml min^{-1}; temperature; ambient; detector: UV 254 nm. Compounds: (1) 2-phenylethylbromide, (2) 1,4-diphenylbutane, (3) phenetole, (4) nitrobenzene, (5) trans-chlorostilbene oxide, (6) sudan red 7B. (From Kromidas, S., *HPLC Made to Measure*. Wiley-VCH Verlag GmBH & Co. KGaA, 2006. With permission.)

HPLC, where the hydrophobicity of the molecule is the predominant determinant of retention differences. When the molecular structure is known, the separation conditions in normal-phase HPLC can be estimated from the solvent selectivity. A small-scale thin-layer liquid chromatographic separation is often a good tool to find a suitable eluent. When a silica gel column is used, the formation of a monolayer of water on the surface of the silica gel is an important technique. A water-saturated, very nonpolar solvent should be used as the base solvent, such as water-saturated *n*-hexane or isooctane.

8.4.1 Classification of Solvents

The most practical method for finding a good eluent for normal-phase HPLC is a trial experiment using thin-layer chromatography (TLC). When a rough separation is achieved by TLC and the maximum R_F value is less than 0.5, the selected solvent is a good candidate to be a suitable eluent for column HPLC. The modification of components depends on the surface activity, that is, the water content, and the surface area of the silica gel.

The selection of the solvent can be based on the solvent properties, classified according to polarity, hydrogen bonding, and solubility parameters. Snyder has classified solvents according to their hydrogen-bonding-acceptor, hydrogen-bonding-donor , and dipole properties. Water and chloroform are classified in the same group; however, these solvents are not miscible. The method for identifying a good developing solvent for TLC is also applicable to HPLC. First, a solvent in which the analyte is very soluble, based on the concept of "like dissolves like" developed by Freizer, must be selected, then a solvent in which the analyte is not soluble is selected; however, these solvents have to be miscible. The eluent is thus a mixture of solvents for the analyte.

The selection of a solvent is based on the solvent strength experimentally obtained on silica gel and alumina. The order of solvent strength and the adsorption parameter on alumina are

not parallel. This indicates that solvent strength cannot be directly related to solvent selection in HPLC.

8.4.2 Preparation of the Eluent

"Like dissolves like" is the basic concept for the selection of solvents in the eluent for HPLC. Controlling the solubility of analytes is the key to success. If the selected solvent or mixture of solvents does not interfere with detection, it is a good eluent. The selection of a suitable solvent for low-wavelength-absorption detection and postcolumn-derivatization detection is important to obtain highly sensitive detection. The selection of a volatile solvent is the key for preparative-scale HPLC and for mass spectrometric detection.

The selection of the solvent is based on the retention mechanism. The retention of analytes on stationary-phase material is based on physicochemical interactions. The molecular interactions in TLC have been extensively discussed and are related to the solubility of solutes in the solvent. The solubility is explained as the sum of the London dispersion (van der Waals force for nonpolar molecules), repulsion, Coulombic forces (compounds form a complex by ion–ion interaction; e.g., ionic crystals dissolve in solvents with a strong conductivity), dipole–dipole interactions, inductive effects, charge-transfer interactions, covalent bonding, hydrogen bonding, and ion–dipole interactions. The steric effect should be included in the above interactions in HPLC separation.

One component of the eluent should have properties similar to those of the analytes, and this solvent is diluted by another solvent to control the retention time. The basic idea can be understood from the chromatographic behavior of phthalic acid esters and polycyclic aromatic hydrocarbons (PAH). This approach can be applied to the separation procedure for a variety of stationary-phase materials, including silica gel, polystyrene gel, and ion exchangers. The elution order of PAHs is based on the number of double bonds, with an increased number of double bonds leading to longer retention time in both normal- and reversed-phase HPLC. These elution orders are always observed on a variety of stationary-phase materials.

The retention time can be modified by changing the eluent components or the ratio of the solvent mixture. Increasing the solubility in the eluent shortens the retention time in both normal- and reversed-phase HPLC. Increasing the alkyl-chain length of analytes makes them more similar to alkanes. The analyte becomes more hydrophobic (lipophilic) and hence soluble in *n*-hexane but not in water. The solubility of both short- and long-alkyl-chain compounds is good in ethanol, which is miscible with *n*-hexane and water. The addition of ethanol to the eluent makes the retention time shorter in both normal- and reversed-phase HPLC. This phenomenon can be observed in the chromatographic behavior of aliphatic acids. However, the solubility of PAHs is poor even in ethanol; therefore, the elution order is always the same in both normal- and reversed-phase HPLC.

In normal-phase HPLC, the elution order of benzene derivatives on silica gel is alkylbenzenes with a long alkyl chain < alkylbenzenes with a shorter alkyl chain < benzene < fluorobenzene < chlorobenzene < cyanobenzene < nitrobenzene < < aniline < phenol < benzoic acid. The elution order of aniline derivatives is dinitroaniline < benzene < anisidine < chloroaniline < nitroaniline < phenylenediamine < aminophenol. This elution order is also observed for anion exchangers. Alkanes are almost unretained. Alkenes are retained according to the number of double bonds. The elution order for *cis* and *trans* isomers is *cis,cis* < *cis,trans* < *trans,trans.*

In other interactions the surface silanol groups of silica gel can form hydrogen bonds, and an alumina surface can form hydrogen bonds and a charge-transfer complex. However, such molecular interactions are caused by positive- and negative-charge sites, as in Lewis acid–base interactions. The chloro ion of chloride-form anion exchangers may form hydrogen bonds with aniline and phenol. Silver ions of silver-form cation exchangers form charge-transfer complexes with the *n*-electrons of alkenes and aromatic compounds.

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9 Separation of Ionic Analytes: Reversed-Phase, Ion-Pair, Ion-Exchange, and Ion-Exclusion HPLC

Monika Waksmundzka-Hajnos and Łukasz Cieśla

CONTENTS

9.1 INTRODUCTION

Natural samples are usually highly complex mixtures containing compounds of different chemical structures and physicochemical properties. Except for neutral compounds, ionized and ionizable compounds occur very often in real botanical samples. Among natural compounds there are numerous acidic substances containing carboxylic groups or slightly ionized hydroxyl groups attached to an aromatic ring, for example, phenolic acids, some flavonoids, and tannins or glucuronic acids. Also, ionizable bases can be encountered in the analysis of natural samples, for example, alkaloids or heterocyclic amines. The aforementioned substances are analyzed often not only because of their common occurrence in plant extracts but also because of their wide spectrum of pharmacological activities. For example, phenolic acids were proved to have biligenic, cholagogue, hypocholesteremic, hypolipidemic, antibacterial, or antifungal properties [1]. Alkaloids are among the most potent herbal drugs, and many of them exhibit poisonous properties. They are used, for example, as spasmolytic, antibacterial, hypotensic, diuretic, analeptic, antifungal, or anticancer drugs [2].

HPLC separation of ionic samples tends to be more complicated than separation of neutral compounds because the former are normally weak acids or bases and are present in aqueous solutions both in ionized and unionized forms [3]. These forms interact differently with the active sites of the adsorbent, which leads to problems in their analysis.

This chapter describes the retention dependence of ionic samples, in reversed-phase HPLC (RP-HPLC) systems, on such conditions as pH, buffer type and concentration, polar modifiers, and ion-pair reagents. Different HPLC methods that are commonly used in the analysis of ionic compounds (RP, ion-pair, ion-exchange, and ion-exclusion HPLC) are described.

9.2 ACIDIC AND BASIC ANALYTES

Aqueous mobile phases are commonly used in RP-HPLC systems; thus, a given acidic or basic compound exists in ionized and nonionized forms. The capacity factor of a solute is given by the following equations.

For acidic compounds,

$$k = k_0\ (1 - F^-) + k_{-1}\ F^-, \tag{9.1}$$

and for basic compounds,

$$k = k_0\ (1 - F^+) + k_1\ F^+, \tag{9.2}$$

where k_0, k_{-1}, and k_1 refer to k values for nonionized and ionic forms, while F^- and F^+ are the fractions of ionized solute molecules:

$$F^- = 1 / \{1 + ([H^+] / K_a)\} \tag{9.3}$$

$$F^+ = 1 / \{1 + (K_a / [H^+])\} \tag{9.4}$$

The data obtained on the basis of these equations remain in the agreement with experimental values for a wide pH range [4–6]. However, considerable deviations have been observed for pH values either $<< pK_a$ or $>> pK_a$. This can be attributed to several factors, including

- The presence of silanols or metal contaminants that are responsible for any nonsolvophobic interactions with a solute
- Changes of pK_a values as a function of ionic strength
- Formation of ion-pairs with ionized buffer species
- Changes in the sorption properties of the stationary phase as a result of the changing ionization of silanols, and so on

Computer programs based on the Equations 1 through 4 are able to predict retention and resolution of ionic samples as a function of pH. However, predicted retention values are usually more accurate for acidic than for basic solutes, due to silanol effects, which are more significant for basic compounds.

Changing the pH values of the mobile phase strongly influences the retention of ionic compounds, as can be seen in Figure 9.1 for benzoic acid derivatives [7]. Changing the pH values can even decrease the retention factors of acids and bases by a factor of 10. As pH increases, RP-HPLC retention for an acid decreases and retention for a base increases. In order to maintain a reasonable k range for the resulting separation ($1 < k < 20$), it is necessary to combine pH optimization with variation of the solvent strength [8,9]. Under such conditions the capacity factor of an ionizable compound is a function of pH and the volume fraction of the organic modifier in the mobile phase (φ):

$$k = f([H^+], \varphi). \tag{9.5}$$

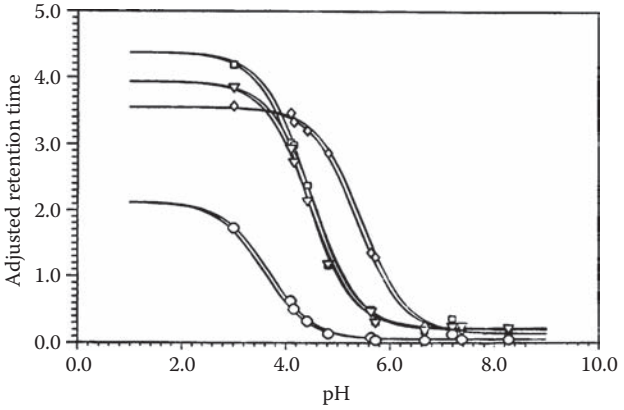

FIGURE 9.1 Adjusted retention times of benzoic acids in 60% methanol relative to the mobile-phase pH. Rhombus, benzoic; circle, 2-nitrobenzoic; triangle, 3-nitrobenzoic; square, 4-nitrobenzoic. (Reprinted from Roses, M., Canals, I., Allemann, H., Siigur, K., and Bosch, E., *Anal. Chem*., 68, 4094–4100, 1996. With permission.)

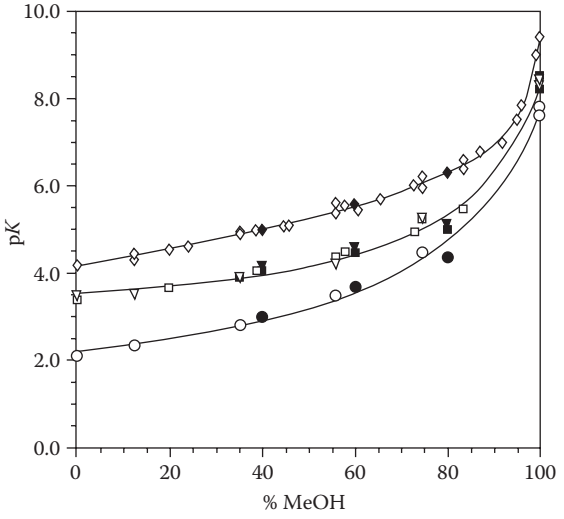

FIGURE 9.2 Variation of the pK_a values of acids based on the methanol content of the mobile phase. Rhombus, benzoic; circle, 2-nitrobenzoic; triangle, 3-nitrobenzoic; square, 4-nitrobenzoic. (Reprinted from Roses, M., Canals, I., Allemann, H., Siigur, K., and Bosch, E., *Anal. Chem*., 68, 4094–4100, 1996. With permission.)

Marques and Schoenmakers provide a detailed study of this problem [10]. The authors propose the following equations for the prediction of acidic and basic compounds' capacity factors:

$$k = \frac{k_0 + k_{-1}K_a / [H^+]}{1 + K_a / [H^+]}, \quad \text{for acids, and} \tag{9.6}$$

$$k = \frac{k_0 + k_1[H^+]/K_a}{1 + [H^+]/K_a}, \quad \text{for bases.} \tag{9.7}$$

However, the situation is much more complicated, as k_0, k_{-1}, k_1, and K_a values vary with the concentration of the modifier in the aqueous mobile phase (see Figures 9.2 and 9.3) [7,11]. To optimize the

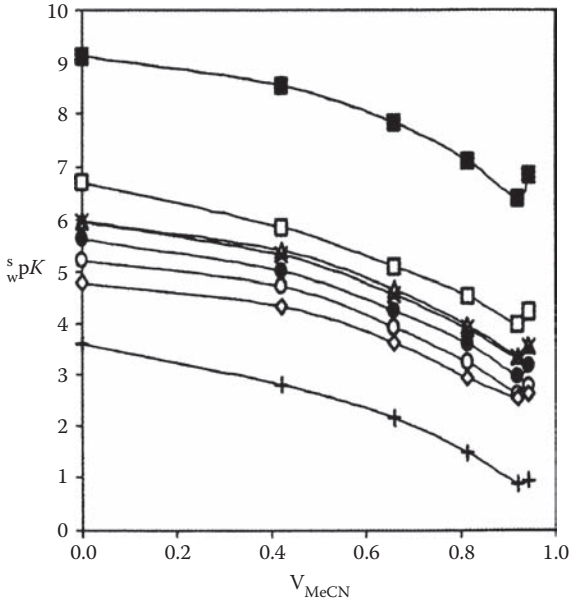

FIGURE 9.3 Variation of pKa values depending on the volume of acetonitrile in acetonitrile–water mobile phase. + , 3-acetylpyridine; ◇, 3-hydroxypyridine; O, pyridine, ●, 3-methylpyridine; x, 2-methylpyridine; Δ, 4-methylpyridine; □, 2-aminopyridine; ■, 4-aminopyridine. (Reprinted from Espinoza, S., Bosch, E., and Roses, M., *Anal. Chim. Acta*, 454, 157–166, 2002. With permission.)

retention process in such complicated systems, Marques and Schoenmakers had two approaches for weak acids [10]:

1. At constant pH they described k_0 and k_1 as a function of the concentration of the organic modifier in the mobile phase:

$$k = \delta + \frac{k_0(\varphi) + k_{-1}(\varphi)K_a(\varphi)/[H^+]}{1 + K_a(\varphi)/[H^+]} \tag{9.8}$$

2. The authors assumed a quadratic relationship between the capacity factor and the concentration of the organic modifier in the mobile phase:

$$\ln k = A + B\varphi + C\varphi^2, \tag{9.9}$$

where A, B, and C are sigmoidal, linear, and quadratic functions of $[H^+]$.

The authors obtained a whole set of different theoretical equations, which they checked practically, and they chose the models giving results similar to the data they had obtained practically. When the percentage of organic modifier in the mobile phase increases, the dielectric constant of the medium and the activity coefficients cannot be neglected. In such a case, retention of ionic solutes may be predicted from the equation

$$t_R = \frac{t_{R(HA)}\gamma_A - \cdot 10^{pK_a - pH} + t_{R(A^-)}}{\gamma_A - \cdot 10^{pK_a - pH} + 1}, \tag{9.10}$$

where $t_{R(HA)}$ is the retention time of neutral acid HA, $t_{R(A^-)}$ is the retention time of the anionic base, and t_R is the measured retention time [7].

All the preceding equations, which assume that the retention of ionic solutes depends only on the pH and mobile-phase composition, may be acceptable only for the neutral form of the solute but not for the ionic species. The retention of such compounds depends mostly on processes such as ion pairing with other ions, solvophobic effects of the ionic strength, and co-ion exclusion resulting from ionization of the residual silanol groups on the adsorbent surface [8].

9.3 REVERSED-PHASE SEPARATION OF IONIC ANALYTES

RP-HPLC is usually the best starting point in the analysis of ionic compounds, due to its simplicity, freedom from problems, and better column performance [3]. The optimization in RP separation and the selectivity control of ionic samples can be performed similarly as for nonionic compounds by varying the solvent strength to obtain a satisfactory k range ($1 < k < 10$), varying the pH, or changing the column type (C8, C18, phenyl, cyano) [12]. Figure 9.4 presents the dependence of selected acids' retention on an octadecylsilica column (ODS) column with a changing amount of organic modifier (methanol).

9.3.1 pH, Buffer Type, and Concentration

Whenever ionic samples are analyzed, the addition of proper buffer is advised. It is important to choose a suitable buffer carefully by taking several properties into consideration: buffer capacity,

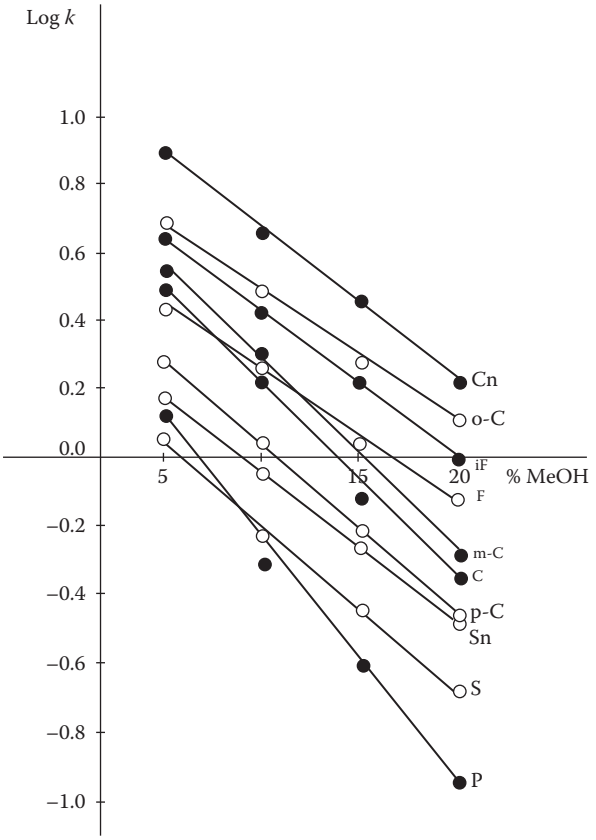

FIGURE 9.4 Plots of log k vs. the percentage of methanol in a mobile phase containing 0.01 M tetraethylammonium column (TEA–Cl) and 0.005 M phosphate buffer at pH 7.17. Column: 10×3.8 cm, 10 µm ODS. Acids: Cn, cinnamic; o-C, *o*-coumaric; i-F, isoferulic; F, ferulic; m-C, *m*-coumaric; C, caffeic; p-C, *p*-coumaric; Sn, sinapinic; S, syringic; P, protocatechuic. (Reprinted from Bieganowska, M.L., Petruczynik, A., and Doraczyńska-Szopa, A., *J. Liq. Chromatogr.*, 13, 2661–2676, 1990. With permission.)

ultraviolet (UV) absorbance, solubility, stability, and interactions with the sample and chromatographic systems [6]. The buffer is effective in controlling pH in the range pK_a ± 1.5, when buffer ionization occurs. The greater the buffer concentration, the greater its capacity. However, higher buffer concentrations may lead to problems in solubility (the salting-out effect). Therefore, a buffer concentration of 10–50 mM is advised, and 25 mM is the best starting point. Commonly used buffers (e.g., phosphate buffer) are more soluble in aqueous methanol than in eluents containing acetonitrile or tetrahydrofuran as organic modifiers.

RP separations are usually performed using silica-based columns, which are stabile in the pH range 2–8. Therefore, the following buffers are commonly used: phosphate buffer (2.1–3.1 and 6.2–8.2), acetate buffer (3.8–5.8), citrate buffer (2.1–6.4), and carbonate buffer (3.8–4.8). The marginal buffer capacity may result in less reproducible separations of ionic compounds, as retention may change from run to run and distorted peaks may appear.

The buffer should transmit light to enable carrying out UV detection; this is especially important if low-UV detection is to be performed (buffers suitable for low-UV detection are phosphate, carbonate, and ammonia). Whenever a buffer is added to the mobile phase, the proper selection of organic modifier is of crucial importance due to differences in buffer salts' solubility in mixtures of water and organic solvents. Usually, aqueous methanol is recommended as the starting point. Mobile-phase pH may change if volatile buffers are used, such as carbonate buffer, due to loss of carbon dioxide. Some buffers may also change the separation conditions; for example, ion pairing can occur in the case of eluents containing trifluoroacetate buffers with cationic samples.

9.3.2 Solvents

Three main solvents are used as organic modifiers in RP-HPLC: acetonitrile, methanol, and tetrahydrofuran. However, other solvents may also be incorporated into the mobile phase. Relative solvent strengths are as follows: water < methanol < acetonitrile < ethanol < tetrahydrofuran < propanol. Acetonitrile is the best initial choice of organic solvent for the mobile phase, as acetonitrile–water mixtures can be used with UV detection at low wavelengths [6]. Due to lower viscosities, low-column pressure as well as a higher plate number is obtained. The next-best organic solvent is methanol, followed by tetrahydrofuran. Some problems may appear in method development in case of tetrahydrofuran use: slower column equilibration or problems with detection in the lower UV range. This solvent also reacts with oxygen, which may lead to irreproducible results. However, some separations may benefit from the addition of tetrahydrofuran, as unique selectivity can be obtained.

A very important factor in RP-HPLC method development is the fact that organic modifiers change the pK_a value [7,11]. In eluents containing larger amounts of an organic modifier, the activity coefficients decrease, and its influence on pH and pK_a values cannot be neglected. It should be a rule of thumb to adjust the pH of the mobile phase before adding the organic solvent [12]. Otherwise, the obtained results may appear to be irreproducible, as electrode response tends to drift. The problem of the retention factor as the combined function of the pH and modifier concentration in the aqueous mobile phase was analyzed by several researchers [10,13–18].

9.3.3 Silanol Blockers and Ion-Suppressing Agents

In the case of basic samples, further problems appear due to the interaction of underivatized free silanols with ionic compounds [19]. It appears to be due to retention by an ion-exchange process that involves protonated bases and ionized silanols [6]:

$$BH^+ + SiO^-K^+ \rightarrow K^+ + SiOBH^+$$

$$H^+ + SiO^-K^+ \rightarrow K^+ + SiOH$$

This case leads to increased retention, band tailing, and column-to-column irreproducibility [20].

These silanol interactions may be minimized by choosing appropriate experimental conditions [21]. Silanol interactions may be reduced by selecting a column that is designed for basic samples with a reduced number of very acidic silanols. The use of a low-pH mobile phase (2.0<pH<3.5) minimizes the concentration of ionized silanols due to suppression of their ionization [22]. HPLC separation of alkaloids with the use of a low-pH buffer can be seen in Figure 9.5. The use of a high pH (>7.0) is also recommended, as the ionization of weak bases is suppressed, thus eliminating ion interactions with acidic silanols. A higher buffer concentration (>10 mM) and proper cations that are strongly held by the silanols (e.g., triethylammonium$^+$, dimethyloctylammonium$^+$) block sample retention. Successful analysis of basic compounds may also be obtained after the incorporation of amines into that mobile phase, as these compete with the analytes for column silanol sites.

The addition of basic silanol-blocking agents causes two effects, depending on the concentration of the blocking molecules: At lower concentrations they are responsible for blocking free silanol sites, leading to a decrease in the analyzed bases' retention (the blockage of the ion-exchange interactions). At higher concentrations, they cause an increase in base retention because of the suppression of basic compounds' dissociation. A detailed study of the influence of different silanol blockers on the retention of basic compounds was presented by Petruczynik [23]. Figure 9.6 shows a chromatogram of the separation of selected alkaloid standards, with the use of diethylamine as a silanol blocker.

In a typical ion-suppression technique, the ionization of a weak acid or the protonation of a weak base is suppressed by adjusting the pH of the mobile phase. Separation is then achieved on an RP column using methanol or acetonitrile and an aqueous buffer solution as the mobile phase. By buffering the mobile phase in the apparent pH 2–5, weakly acidic solutes can be retained on a reversed-phase column. In addition, weakly basic substances can also be separated on a similar column by ion suppression if the pH of the mobile phase is maintained in the range 7–8 [24]. In the analysis of acidic compounds it is also a common procedure to add organic acids, such as formic or acetic acid, into the mobile phase instead of a buffer. It suppresses the dissociation of the analyte, which becomes more lipophilic, thus more strongly retained [25]. Figure 9.7 presents the separation of phenolic acids with the application of acetic acid as an ion-suppressing agent.

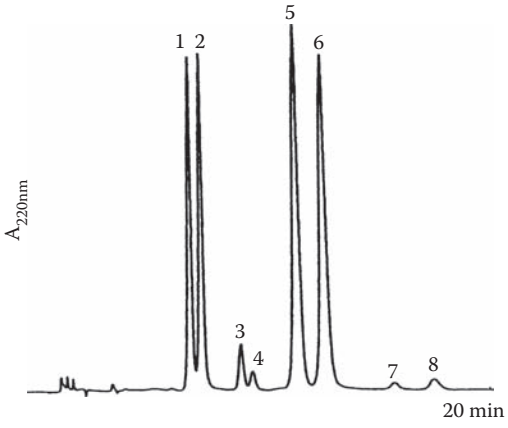

FIGURE 9.5 HPLC separation of alkaloids on the LiChrosorb RP-8 column. Eluent: 15% acetonitrile in 0.1 M phosphate buffer, pH 3. Constituents: 1, cinchonine; 2, cinchonidine; 3, dihydrocinchonine; 4, dihydrocinchonidine; 5, quinidine; 6, quinine; 7, dihydroquinidine; 8, dihydroquinine. (Reprinted from McCalley, D.V., *J. Chromatogr. A*, 967, 1–19, 2002. With permission.)

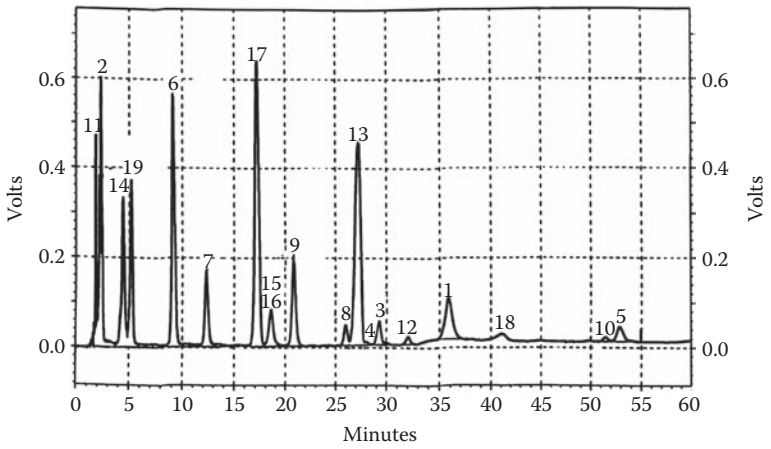

FIGURE 9.6 Chromatogram presenting separation of isoquinoline alkaloid standards' mixture on phenyl column with the use of a mobile-phase gradient: 15–50% acetonitrile + buffer to pH 3.5 + 0.05 M ·l-1 diethylamine (DEA) (time 60 minutes). Solutes: 1, berberine; 2, boldine; 3, chelidonine; 4, unidentified peak; 5, homochelidonine; 6, codeine; 7, dionine; 8, emetine; 9, glaucine; 10, chelerythrine; 11, laudanosine; 12, unidentified peak; 13, paracodine; 14, narceine; 15, noscapine; 16, papaverine; 17, protopine; 18, sanguinarine; 19, tubocurarine. (Reprinted from Petruczynik, A. and Waksmundzka-Hajnos, M., *J. Liq. Chromatogr. Related Technol*., 29, 2807–2822, 2006. With permission.)

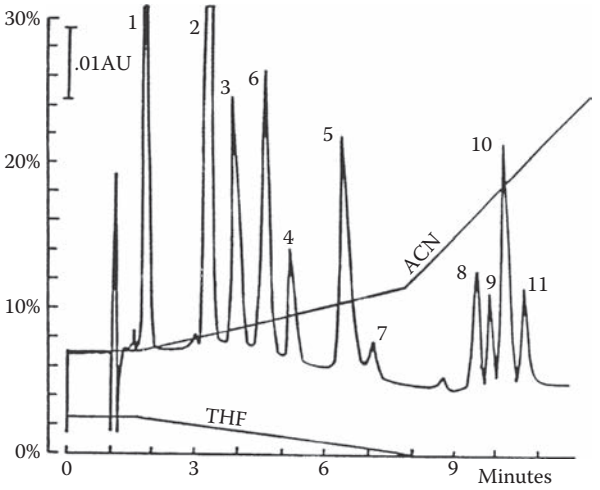

FIGURE 9.7 Separation of phenolic acids by gradient elution with 7–11.6% acetonitrile and 2.5–0% tetrahydrofuran during 0–6 min and 11.6–30% acetonitrile during 6–12 min. All solvents contained 1% acetic acid. Solutes: 1, gallic; 2, protocatechuic; 3, chlorogenic; 4, vanillic; 5, trans-caffeic; 6, syringic; 7, cis-caffeic; 8, trans-*p*-coumaric; 9, cis-*p*-coumaric; 10, trans-ferulic; 11, cis-ferulic. (Reprinted from Dzido, T.H. and Smolarz, H.D., *J. Chromatogr. A*, 679, 59–66, 1994. With permission.)

9.4 ION-PAIR CHROMATOGRAPHY (IPC)

If a successful separation of a sample containing ionic compounds has not been obtained with the use of RP-HPLC, IPC provides an important additional selectivity option [6]. IPC and RP-HPLC share several features. The column and mobile phase used for the separations are generally similar, differing mainly in the addition of an ion-pairing reagent.

There are several theories explaining the mechanism of adsorption in ion-association systems [26–31]. The ion-pair model assumes an association between the sample ion and oppositely charged ion-pairing reagent in a liquid polar mobile phase before its adsorption on the hydrophobic stationary phase [4,32–34]. According to this theory the capacity factor of an ionic solute depends on the kind and concentration of the counterion. The second model of the IPC mechanism is the ion-exchange model [35–39]. It assumes the adsorption of lipophilic counterions on the nonpolar surface of the stationary phase, which then behaves like an ion exchanger. The greater the concentration of the ion-pair reagent and its hydrophobicity, the greater the retention of the chromatographed ionic compounds [40]. Bidlingmeyer et al. proposed an ion-interaction model—a model of a double electric layer [41]. According to this theory the dynamic equilibrium of the lipophilic ion occurs in the double electric layer formed on the sorbent surface. The retention of the analyte is caused by the charge of the double layer formed by the ions of the ion-pairing reagent. Stahlberg et al. proposed the electrostatic model, which assumes the ion-pairing reagent is fully ionized in the applied pH range and influences first of all the retention of the solute's ionized form [42–45].

Except for ion-pair reagent type, concentration, and mobile-phase pH, the retention and selectivity in ion-pair reversed-phase (IP-RP) systems can be controlled by changing the type and concentration of the organic modifier in the aqueous mobile phase [12,46–47]. The retention of solutes decreases as the concentration of the organic modifier increases, and the log k values are linear functions of the volume concentration of the modifier in accordance with the equation

$$\log k = \log k_w - b\, C_{mod}, \tag{9.11}$$

where k_w is the capacity factor for pure water or aqueous buffer solution and b is constant [48].

Changing the buffer type and concentration also may cause variations in the selectivity of the separation. Further variations in selectivity may occur if the stationary-phase type or temperature is changed [3]. Wide ranges of reversed-phase packing materials have been applied as a stationary phase in IPC separations. These materials include neutral polystyrene divinylbenzene (PS-DVB) polymers [49], silica materials bonded with C2, C8, or C18 alkyl groups, and cyano groups [26,50–52].

When compared with RP-HPLC, additional problems may appear in the development of IPC methods. These are the possibility of forming positive or negative artifact peaks, slower column equilibration, difficulties in performing gradient elution (more erratic baseline, less reproducible retention), or poor peak shape in some ion-pair systems [3].

9.4.1 pH and Ion Pairing

Mobile-phase pH is an important determinant ion-pair reversed-phase chromatography [29,53–54]. It should be selected to obtain maximal ionization of solute and ion-pairing molecules to possibly form an ion-pair. For example, for acidic solutes, the pH used is usually in the range 7.0–7.5; higher pH values may destroy a silica-based adsorbent [12]. When $pK_a - 2 < pH < pK_a + 2$, the solute molecules exist in ionic and nonionic forms, and the adsorption of both forms (ionic and nonionic) and ion pairing occur.

When the pH and the ion-pair reagent and its concentration are varied simultaneously, considerable control is achievable over both retention range and band spacing. This is a result of the simultaneous retention of the sample by both reversed-phase and ion-exchange processes.

9.4.2 Ion-Pair Type and Concentration

An ion-pair reagent might cause a very large change in chromatographic properties, enabling the analyte to be moved well away from previously co-chromatographic materials present in the sample that do not interact with the ion-pair reagent [3].

In the case of acidic compounds, the following cationic ion-pairing reagents have been employed: alkylammonium compounds [55,56], organic amines and other basic compounds [57–59], and so on. In the case of basic compounds, anionic ion-pairing reagents are used, such as sulfonic acids, alkyl sulfonates, or other acids, for example, bis(2-ethylhexyl)orthi-phosphoric acid. The situation is much more complicated in the case of samples containing both acidic and basic compounds, where the choice of reagent type (anionic or cationic) is difficult. Usually, using a mixture of ion-pair reagents with opposite charges is discouraged, since the two reagents will associate and hence tend to neutralize each other's effect on the sample. However, the simultaneous use of cetyltrimethylammonium (CTMA) and dodecasulfonate (DS) for separation of basic samples was reported [3]. CTMA was used to reduce the stationary-phase silanols effect. It should be kept in mind that sulfonate reagents strongly increase the retention of positively charged species and strongly decrease the retention of negatively charged species (and vice versa for tetraalkyl ammonium reagents). Any ion-pair reagent may reduce the retention of neutrals, but to a lesser degree [3].

With the greater uptake of ion-pair reagent by the column, the retention mechanism changes from a reversed-phase to an ion-exchange one [12]. More hydrophobic ion-pair reagents saturate the column at a lower mobile-phase reagent concentration. An identical separation can be achieved with different ion-pair reagents. A higher mobile-phase concentration of a less hydrophobic reagent is needed to obtain comparable analyte retention.

The elongation of the alkyl chain of an ion-pair reagent by a single methylene group leads to an increase in the log k value by about 0.2 units [60]. A similar situation is observed when the degree of the amines substitution increases: Introduction of a methyl group to a primary, secondary, or tertiary amine increases the log k value by about 0.4 units [60,61]. In a limited range of concentrations, a linear relationship between log k and log of concentration is obtained [62,63]:

$$\log k = \text{const} - m \log [X], \tag{9.12}$$

where $[X]$ is the mole concentration of the ion-pairing reagent. After the surface is saturated by hydrophobic counterions, a further increase in the concentration does not lead to significant changes in retention [64,65]. Changing the type and concentration of the counterion often causes variations in the selectivity of the separation [46,47].

IPC has been used for example in the analysis of phenolic acids on phenyl column—tetraethylammonium iodide was used as the ion-pair reagent [66]. As far as basic natural substances are considered IPC systems have been applied for example in the analysis of indole [67], pyrrolizidine [68], *Aconitum* [69], or isoquinoline alkaloids [70] (see Figure 9.8). More examples of using IPC to analyze basic compounds can be found in the review by Waksmundzka-Hajnos [19].

9.5 ION-EXCHANGE CHROMATOGRAPHY (IEC)

Nowadays, IEC is applied infrequently compared to other chromatographic methods. Both IPC and IEC have comparable retention mechanisms, but the first method is more convenient due to higher column efficiency, more stable and reproducible columns, and easier control over selectivity and resolution [3]. IEC is recommended when

- The analyzed substances lack chromophores, and conductivity detection is needed;
- A mass spectrometer detector has to be used, as volatile buffers used in IEC fulfill demands associated with the use of this detector;
- The analyzed substances are isolated or purified by HPLC, as it is easier to remove the mobile-phase components applied in IEC separations than ion-pair reagents used in IPC; or
- Multidimensional separations are used, as the aqueous buffer-salt mobile phase used for ion exchange allows direct injection of a sample fraction onto an RP column.

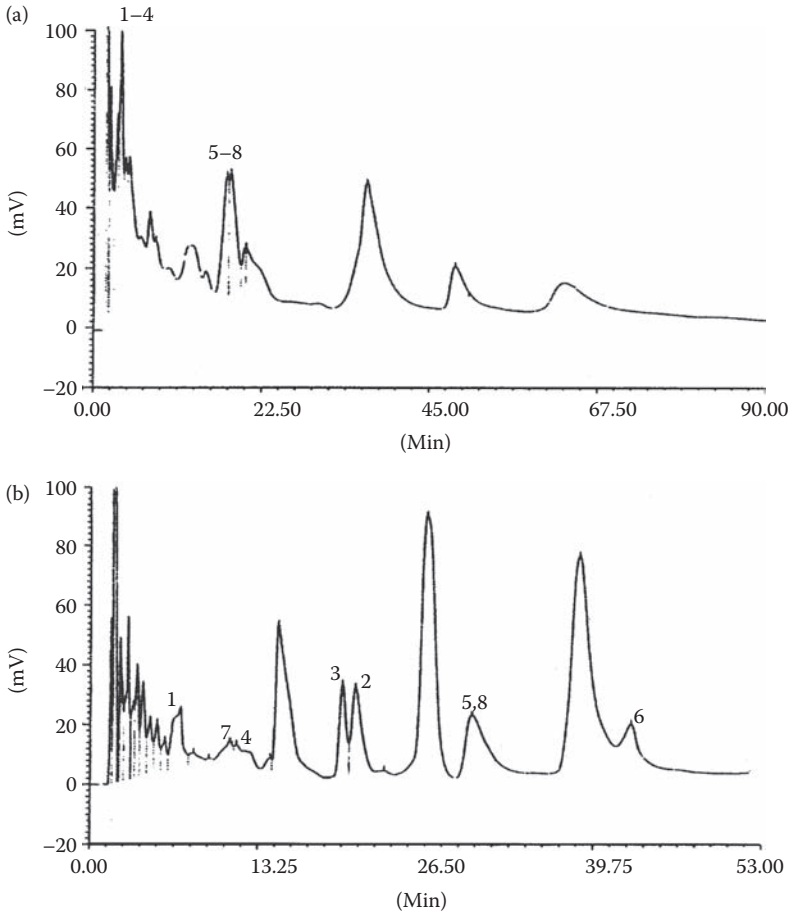

FIGURE 9.8 Separation of alkaloids from *Chelidonium majus* herb on CN-silica column with the use of the following eluents: (A) 20% acetonitrile + buffer to pH 8, (B) 20% acetonitrile + buffer to pH 6 + 0.001 M sulfonic acid sodium (SOS). Solutes: 1, allocryptopine; 2, berberine; 3, chelerythrine; 4, chelidonine; 5, chelilutyne; 6, chelirubine; 7, homochelidonine; 8, protopine. (Reprinted from Petruczynik, A., Gadzikowska, M., and Waksmundzka-Hajnos, M., *Acta Pol. Pharm*., 59, 61–64, 2002. With permission.)

IEC demands the application of anion-exchange columns (stationary phases with attached quaternary ammonium groups) for the separation of organic acids, while weak bases are separated with the use of cation-exchange columns (stationary phases with attached sulfonic or carboxylic groups). There are two subgroups of anion- and cation-exchange columns: weak anion/cation exchangers and strong anion/cation exchangers not suitable for HPLC. The retention of basic (X^+) and acidic (X^-) compounds on such stationary phases (R^- or R^+, respectively) can be presented by the equilibrium of an ion-exchange:

Basic compounds	Acidic compounds
$X^+ + R^- K^+ \leftrightarrow X^+ R^- + K^+$	$X^- + R^+ Cl^- \leftrightarrow X^- R^+ + Cl^-$

where K^+/Cl^- plays the role of counterion in the mobile phase, and the effect of its concentration on the retention of a sample ion of charge z is

$$k = \frac{\text{constant}}{(\text{counterion concentration})^z}. \tag{9.13}$$

9.5.1 Eluents, pH, and Buffer Type

The retention of ionic solutes can be controlled by changing the pH, which is a preferred way to change selectivity in ion-exchange separations. In IEC only the ionized form of a compound is retained on the oppositely charged solvent. The increase in pH leads to stronger ionization and retention of the acidic sample, while the decrease in pH causes greater retention of basic compounds. In IEC, buffers are often used to attain a desired pH during the ion-exchange operation [71].

Another way of changing retention in IEC systems is to use different counterions. The relative strength of various displacers in the anion- and cation-exchange process is as follows [3]:

- For the anion-exchange process,

$$F^- \text{(weak)} < OH^- < \text{acetate} < Cl^- < SCN^- < Br^- < CrO_4^- < NO_3^- < I^- < SO_4^{2-} < \text{citrate}$$

- For the cation-exchange process,

$$Li^+ \text{(weak)} < H^+ < Na^+ < NH_4^+ < K^+ < Rb^+ < Cs^+ < Ag^+ < Mg^{2+} < Zn^{2+} < Co^{2+} <$$

$$Cu^{2+} < Cd^{2+} < Ni^{2+} < Ca^{2+} < Pb^{2+} < Ba^{2+} \text{ (strong)}$$

Acid derivatives may also be separated on a zirconia surface with anion-exchange properties. Blackwell presented a separation of benzoic acid derivatives on zirconia columns [72,73]. IEC has mainly been used for purifying or isolating ionic compounds (solid-phase extraction [SPE] procedure preceding HPLC analysis). Ion-exchange SPE was performed, for example, on strong cation-exchange, SCX-benzenesulfonic acid cartridges [74]. However, several papers present the use of IEC for the separation of ionic solutes; for details see References [12,19].

9.5.2 Organic Solvents

Sometimes, organic modifiers such as methanol, acetonitrile, or tetrahydrofuran are added in IEC. This causes decreased retention of ionizable compounds [12] .

9.6 ION-EXCLUSION CHROMATOGRAPHY

Ion-exclusion chromatography (also known as ion-exclusion partition chromatography, Donnan exclusion chromatography, or ion-moderated partition chromatography) is based on the separation of partially ionized species on strong anion-exchange or strong cation-exchange stationary phases, with Donnan exclusion of the analytes from the charged stationary phase being the basic separation mechanism [75].

Several parameters influence retention in ion-exclusion systems, such as the degree of ionization of the analyte, the eluent concentration and its pH, and the presence of organic solvents in the eluent. Other parameters influencing the separation in the investigated systems have been comprehensively described elsewhere [75].

Anionic samples are separated on fully sulfonated column packings (e.g., polystyrene-divinylbenzene [PS-DVB]), while in the case of cationic solutes, usually fully functionalized resins with quaternary ammonium groups are applied. The use of an unmodified silica gel column with sulfuric acid eluent [76] or silica gel modified with aluminum [77] is also reported for the ion-exclusion chromatographic separation of hydrophobic carboxylic acids. There are also reports describing the use of weakly acidic cation-exchange columns for the separation of acidic compounds. Aqueous solutions of tartaric or sulfosalicylic acids were used as eluents [78]. For analysis of weak acids, so-called vacancy ion-exclusion chromatography has been proposed [78]. In this method a mixture of aliphatic carboxylic acids was used as the eluent, and water was injected into the separation column as the sample.

Commonly used eluents include water; sodium hydroxide aqueous solution; a mixture of glycerol–water; aqueous eluents containing methanol, ethylene glycol, glycerol, erythritol, xylitol, fructose, glucose, sorbitol, and sucrose (the separation of weak bases); aqueous solutions of strong acids such as sulfuric acid [79]; polyvinyl aqueous solution [80]; *n*-butanol [81]; and benzoic acid in aqueous methanol [82] (for the separation of weak acids). The addition of various organic compounds, such as alcohols, acetonitrile, and sugar alcohols, to an acidic eluent is also carried out in order to improve peak shapes and reduce retention volumes for hydrophobic carboxylic acids [83].

Acidic compounds interact with the sulfonated PS-DVB copolymer. The dissociated fraction of the analyte is repelled from the vicinity of the Donnan membrane, while the protonated fraction penetrates the membrane and enters the occluded fraction of the eluent. The higher the protonated fraction is, the longer the retention time of the analyte will be. However, the retention mechanism of ion-exclusion is still discussed; for more detailed information see Reference [75].

In the case of ion-exclusion chromatography, UV and/or conductometric detection can be performed. The second mode is used for solutes lacking a proper chromophore. When conductivity detection is applied, the acidic or basic eluents cause a rather high background conductance and reduce the ability to detect sample acids or bases [80]. In such a case, the use of eluents with low-background conductivity (e.g., elution with polyols or sugars) is advised. A combination of ion-exclusion chromatography with atmospheric pressure chemical ionization–mass spectrometric detection has also been reported [82].

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10 Gradient Elution and Computer-Assisted Method Development

Mirosław Hawrył

CONTENTS

10.1 ISOCRATIC ELUTION AND GRADIENT ELUTION, THE GENERAL ELUTION PROBLEM

Isocratic elution is a mode of high performance liquid chromatography (HPLC) development in which the concentration of the mobile phase is constant during the whole chromatographic process. This method is applied in the case of uncomplicated mixtures, when the separated compounds' retention factors are different but compatible.

Complex mixtures that contain many (e.g., 15 or more) components are difficult to separate in an isocratic run. If a weak solvent is used, all peaks may be satisfactorily separated, but the retention times of the last compounds are too long for the chromatographic analysis to be considered optimal (Figure 10.1a). In contrast, if a stronger solvent is applied, the time of analysis is acceptable, but

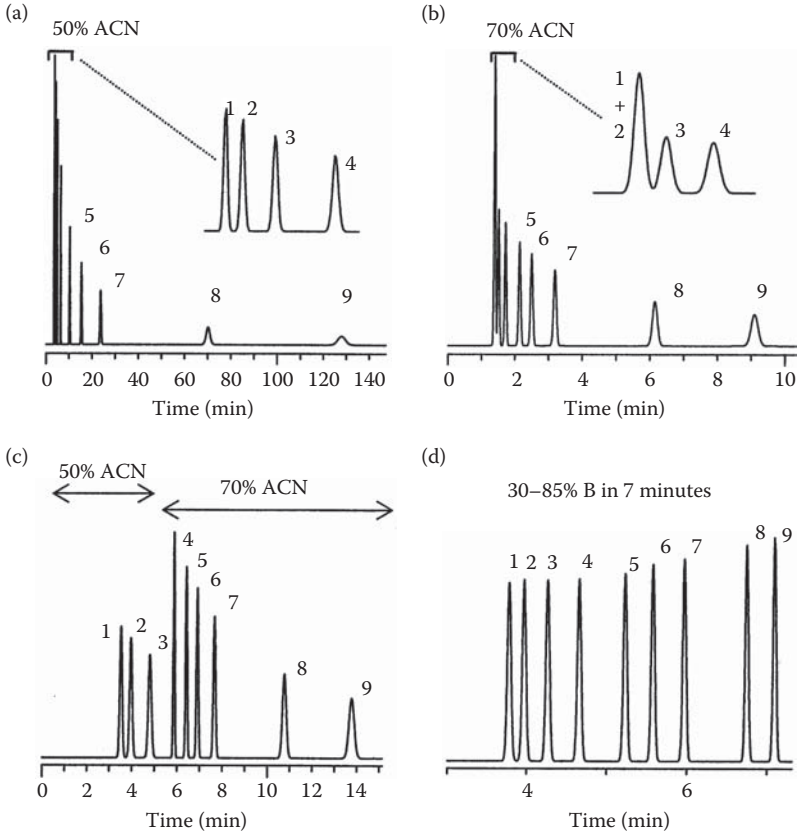

FIGURE 10.1 Illustration of the general elution problem and its solution. The sample is a mixture of herbicides (equal areas for all peaks). (a) Isocratic elution using 50% acetonitrile (ACN):water as the mobile phase; 150×4.6 mm C_{18} column (5 µm particles), 2.0 mL/min, ambient temperature. (b) Same as (a), except 70% ACN and water; (c) Same as (a), except stepwise elution with 50% ACN for 5 minutes, followed by 70% ACN for 10 min. (d) Same as (a), except gradient elution: 30–85% ACN in 7 minutes. (Reprinted from Snyder, L.R. and Dolan, J.W., *High-Performance Gradient Elution: The Practical Application of the Linear-Solvent-Strength Model*, J. Wiley & Sons, Hoboken, NJ, 2007, 2. With permission.)

some of the peaks are not separated completely (Figure 10.1b). The situation shown in Figure 10.1a and b is the "general elution problem," that is, the inability of a single isocratic separation to provide satisfactory separation within a reasonable time for compounds with a wide range in retention (peaks with very different retention factors k). In this case, the gradient mode can be applied. Gradient elution is the mode of chromatographic development in which the concentration of the mobile phase changes during the chromatographic process.

The "general elution problem" can be resolved in several ways:

1. Solvent gradient
2. Column switching (stationary-phase gradient)
3. Temperature and flow-rate gradients

Figure 10.1c shows stepwise elution with a weaker solvent for 5 minutes followed by stronger solvent for 10 minutes. All peaks are separated within an acceptable time, but sizable variations in peak spacing still exist. Sometimes, a two-step gradient is an effective method of separation, but this method is difficult to reproduce experimentally and "peak splitting" (the appearance of two peaks for a single compound) and "ghost peaks" can occur.

Gradient elution is the best method for separating complex mixtures, for example, those obtained from plant materials. Figure 10.1d shows the gradient separation of nine compounds (the same ones as in Figure 10.1a, b, and c) using continuous gradient elution. All peaks are completely separated in a total run time of 7 minutes. The peaks have approximately constant widths and comparable detection sensitivity.

Reversed-phase systems are the most popular ones for HPLC separations. However, in some cases, normal-phase systems are used. Some aspects of the optimization of the gradient in normal-phase HPLC systems have been studied [1,2].

In most cases isocratic elution is suitable only for the analysis of samples with a narrow retention range. Gradient elution can be used to separate samples with a wide retention range and is more suitable for the establishment of methods for analyzing complex samples, such as phytochemical, biological, and environmental samples. Linear gradient elution has the advantage of easy operation and good separation of most components in a suitable period of time. Snyder et al. [3–22] comprehensively studied the optimization of separation conditions in HPLC gradient systems, especially with respect to the selectivies of various types of columns and the influence of temperature and solvent strength on the gradient separations, as well as the use of computer-aided methods.

Some aspects of pH gradients in ion chromatography were studied by Kaliszan [23] and Bolanca [24]; ghost peaks in gradient reversed-phase HPLC, stationary-phase effects, and other parameters were also described [25–32]. Multilinear gradient optimizations and problems related to them were discussed by Concha-Herrera [33]. Preparative gradient chromatography was reviewed by Seidel-Morgenstern [34].

Since mobile-phase compositions have different selectivities for different components in the analyzed sample, in many cases satisfactory results for multicomponent samples cannot be achieved using simple linear gradient elution. The multisegment gradient elution mode is able to make full use of organic modifiers' ability to adjust samples' separation selectivity; however, the optimization procedure is difficult due to the many parameters that can be adjusted [35–37]. The new nonlinear relationships and its applications in gradient elution were also studied [22,28,38].

10.2 SOLVENT GRADIENT

The optimization of gradient elution is performed by searching for the optimal selectivity and efficiency of separation. There are several elements that influence these values: the type of stationary phase, the modifier, the mobile-phase additives, and the temperature. The gradient itself must be selected to give a mobile phase that is initially just strong enough to elute the fastest peaks. Later, its composition is changed by increasing the solvent strength to elute the more retained compounds.

Each gradient is characterized by its "gradient shape." The "gradient shape" is the way in which mobile-phase composition (the percentage of the modifier B by volume in reversed-phase chromatography) changes over time during a gradient separation. There are six basic gradient shapes, which are presented in Figure 10.2.

Most gradients have a linear shape (Figure 10.2a); these are strongly recommended during the initial stages of method development. Concave and convex gradients (Figure 10.2b and c) are useful if the second component elutes much more strongly than the first. In this case, a small addition of B produces a considerable change in the mobile-phase elution strength. These curved gradients were often used in the past. Nowadays, curved gradients have been replaced by segmented gradients (Figure 10.2d). Segmented gradients can provide all the advantages of curved gradients and also furnish a greater control over separation. A gradient delay and a step gradient are also shown, in Figure 10.2e and f.

10.3 COLUMN SWITCHING

The term *column switching* refers to the technique for resolving the general elution problem in which the analyzed sample is initially separated into several fractions about various polarities in the first column (Figure 10.1). The first fraction (the least polar one) is directed to the second column, the middle polar fraction to the third column, and the last (most polar) fraction to the fourth column.

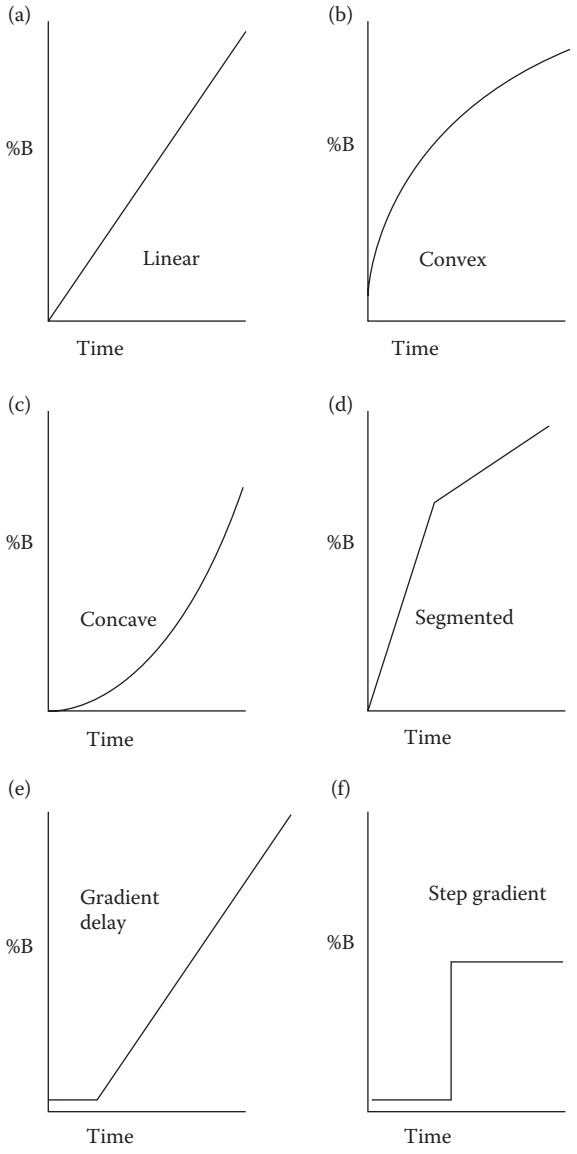

FIGURE 10.2 Illustration of different gradient shapes (plots of the percentage of B at the column inlet versus time). (Reprinted from Snyder, L.R. and Dolan, J.W., *High-Performance Gradient Elution: The Practical Application of the Linear-Solvent-Strength Model*, J. Wiley & Sons, Hoboken, NJ, 2007, 4. With permission.)

The separation is carried out by changing the position of the four-port valve between columns 2, 3, and 4. The change of stationary phase in columns 2–4 causes k values to be maximal in column 2, smaller in column 3, and smallest in column 4. It provides good resolution and the fast elution of various fractions analyzed in particular columns. The general resolution obtained in this method is not much worse than that obtained in gradient elution.

Columns and valves are arranged depending on the analytical problem to be resolved. Various possible column combinations can be applied [39]:

a. Adsorbents with different specific surface areas
b. Reversed phases with different chain lengths

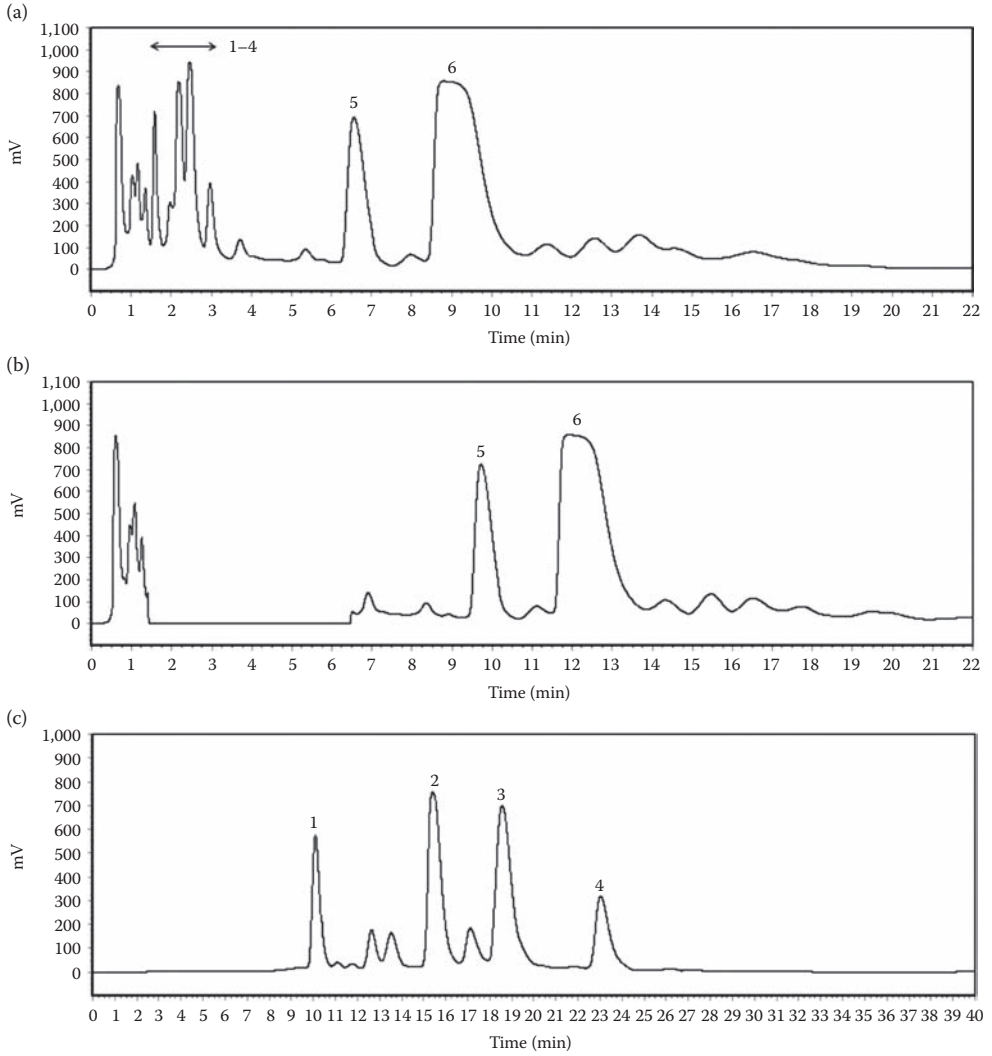

FIGURE 10.3 Chromatograms illustrating the separation of the components in extract from *Gardenia jasminoides* Ellis. The injection volumes were 2.0 mL, with detection at 238 nm. (a) Chromatogram illustrating the separation of all the sample components on a Lichrospher C_{18} (100 × 10.0 mm ID; 5 µm). Mobile phase: 7% acetonitrile (including 0.1% acetic acid); flow rate: 9.8 mL min^{-1}. (b) Chromatogram illustrating the separation of partial sample components on a Lichrospher C_{18} (100 × 10.0 mm ID; 5 µm) in the first dimension. Mobile phase: 7% acetonitrile (including 0.1% acetic acid); flow rate: 9.8 mL min^{-1}. (c) Chromatogram illustrating the separation of four target components on an YMC ODS column (250 × 10 mm ID, 5 µm) in the second dimension. Mobile phase: 7% acetonitrile (including 0.1% acetic acid); flow rate: 4.0 mL min^{-1}; switch volumes: 20 mL. 1 = shanzhiside; 2 = deacetyl-asperulosidic acid methyl ester; 3 = gardenoside; 4 = scandosidemethyl ester; 5 = genipin–1-β-D-gentiobioside; 6 = geniposide. (Reprinted from Zhou, T., Zhao, W., Fau, G., Choi, Y., and Wu, Y., *J. Chromatogr. B*, 858, 296–301, 2007. With permission.)

c. Ion-exchangers of different strengths
d. A combination of anion and cation exchangers
e. A combination of various methods such as ion-exchange and reversed-phase chromatography, size-exclusion and adsorption chromatography, affinity and reversed-phase chromatography, and so on

The equipment and the procedure are quite simple, there is no need of the column regeneration, and all available detectors can be applied. The sensivity of the detection is almost the same as in gradient methods, so the column-switching technique can be used to resolve the general elution problem. Chromatograms illustrating the separation of the components in *Gardenia jasminoides* extract are presented in Figure 10.3.

The comprehensive literature on two-dimensional HPLC and its applications and theory was reviewed by Guiochon [40], and "orthogonality" in reversed-phase liquid chromatography systems was studied by Pellett [41].

10.4 TEMPERATURE AND FLOW-RATE GRADIENTS

Programming of temperature is a method in which the temperature increases during the chromatographic process. In this way, the retention factors of separated compounds decrease, as in gradient elution. The main problem in temperature programming is the reduced resolution caused by changes in eluents' viscosity, which are connected with changes of temperature. Figure 10.4 shows chromatograms of a variety of carotenoids on a C_{30} stationary phase at various temperatures. Nowadays, interest in high-temperature liquid chromatography (HTLC) is observed [42–47].

Flow-rate programming is the technique in which the linear velocity of the eluent is lower at the start of the separation process and then increases during the chromatographic elution. In this way, the resolution is transferred from the finish of the chromatogram (where it is usually not needed) to the initial part of the chromatogram where it is needed. This method guarantees only marginal increases in the resolution in the initial part of chromatogram, and this technique is useless for samples containing many compounds with various polarities. The basic advantage of this method is the simple equipment and experimental technique, and for these reasons flow-rate programming is sometimes used.

10.5 APPLICATIONS OF GRADIENT HPLC IN PHYTOCHEMICAL ANALYSIS

In this chapter some papers are presented to show chromatographic methods and procedures used in the analysis and determination of various groups of biologically active plant compounds. These reviews describe various chromatographic and/or capillary electrophoretic techniques in phytochemical analysis (included gradient HPLC methods). Each paper contains a comprehensive list of references, which can be used in further literature studies. For detailed information about applications of gradient HPLC in the analysis of some groups of phytochemical compounds, see Table 10.1.

10.5.1 Phenolics

The typical system involves reversed-phase liquid chromatography using a C_8 or C_{18} stationary phase or other alkyl chemistry [48]. Columns are commonly 100–300 mm in length with 10 mm, 5 mm, or smaller particles. In most cases gradient elution is used in recognition of the complexity of most samples' phenolic profile. Numerous mobile phases have been employed, but binary systems comprising an aqueous component and a less polar organic solvent such as acetonitrile or methanol remain common. Acids (acetic, formic, trifluoroacetic, or phosphoric acid) are usually added to both components to maintain constant acid concentration during gradient runs. Ternary phases offer greater flexibility and will likely increase in popularity [49]. The separation normally requires 1 h at a flow rate of 1.0–1.5 mL/min.

Detection in HPLC is routinely achieved by ultraviolet (UV) absorption, often using a photodiode array detector (PDA); however, PDA has mostly been used as a convenient multiple-wavelength detector, and its versatility often appears to have been neglected [50]. The hydrophilicity of polyphenols is relative and spans a wide range. Oligomeric procyanidins, for example, are relatively less

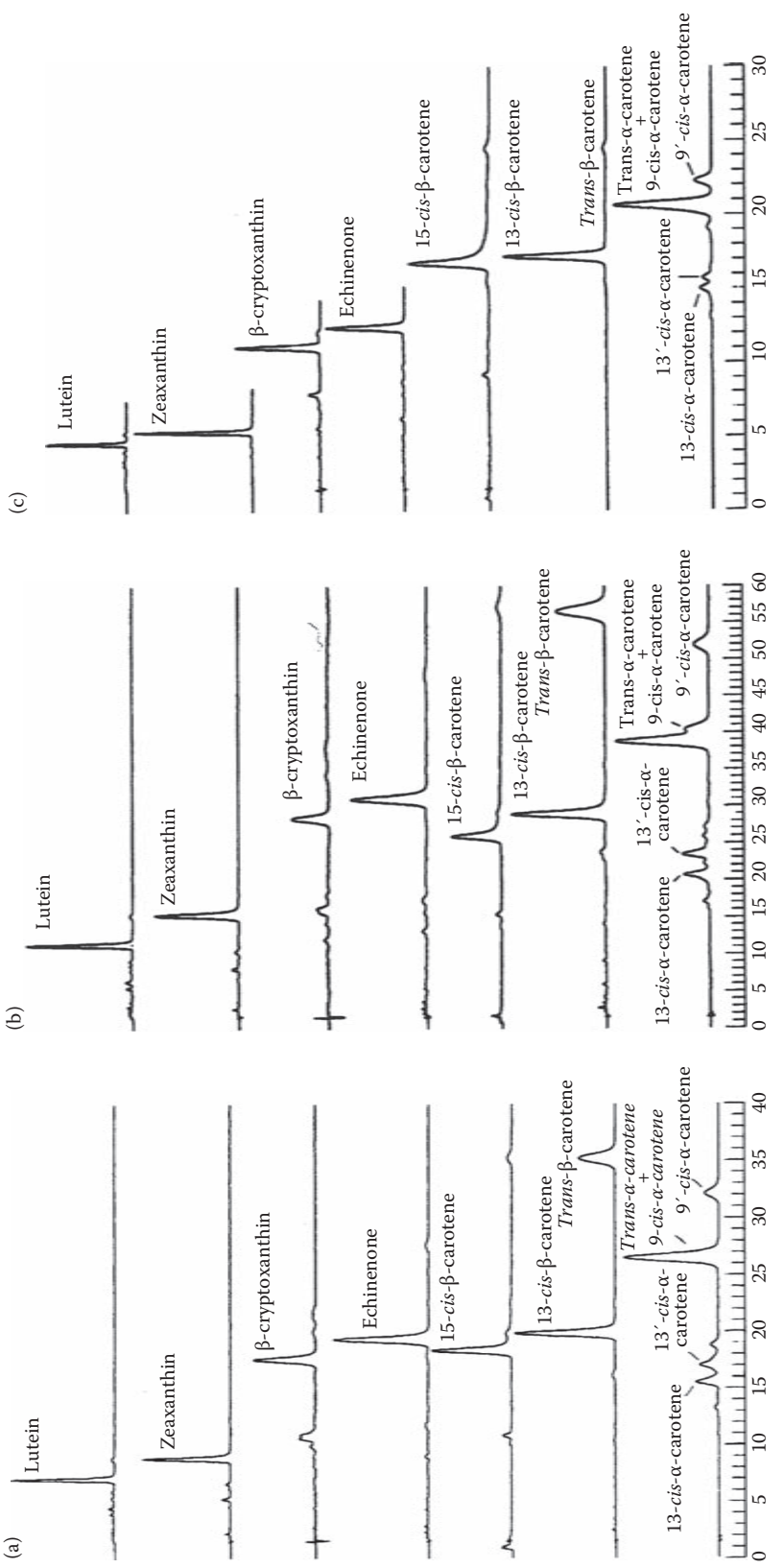

FIGURE 10.4 Chromatogram of a variety of carotenoids on a C_{30} stationary phase at (A) 25°C, (B) 13°C, and (C) 38°C. (Reprinted from Bell, C.M., Sander, L.C., and Wise, S.A., *J. Chromatogr. A*, 757, 29–39, 1997. With permission.)

TABLE 10.1
Applications of HPLC Gradient Development Methods in Phytochemical Analysis

Compounds and Plant Material	Column	Eluents	Gradient Program	Other Conditions	Detection	Ref.
Flavonoids						
Trifolium pratense	C_{18} Hypersil, 200 × 2.1 mm, 5 µm,	(A) Water, (B) MeOH	0–30 min: 0%–100% B; FR 0.2	Temp. 45°C	MS	[154]
Ginkgo biloba *Podocarpus dacrydioides* *Pseudotsuga menziesii*	Various C_{18} columns	I. (A) THF, (B) PA:water (99:1) II. (A) MeOH, (B) PA:water (99:1) III. (A) Citrate buffer, (B) ACN	I. 0–160 min: 5%–100% B; FR 0.8 II. 0–90 min: 5%–100% B; FR 0.45 III. 0–47 min: 45%–74% B; FR 1.0		UV	[155]
Trifolium pratense *Trifolium dubium* *Trifolium repens* *Lotus corniculatus*	SB-C_{18} Zorbax, 250 × 4.6 mm, 5 µm	(A) 10 mM ammonium formate buffer, pH 4.0, (B) MeOH	0–5 min: 30%–45% B; 5–10 min: 45% B; 10–15 min: 45%–50% B; 15–25 min: 50%–55% B; 25–30 min: 55%–60% B; 30–35 min: 60%–80% B; 35–40 min: 80% B; 40–42 min: 80%–30% B; FR 1.0		PDA, UV-FLU, ESI-MS	[156]
Spinacia oleracea	C_{18} Nucleosyl, 250 × 4 mm, 5 µm	(A) water + triethylamine (1 mL/L) + PA 85% (1 mL/L) + KH_2PO_4 (1 g/L), (B) ACN	0–7 min: 20% B; 7–25 min: 20%–25% B; 25–40 min: 25%–55% B; 40–50 min: 20% B; FR 1.0		PDA	[157]
Citrus aurantium	C_{18} Hypersil, 250 × 4.6 mm, 5 µm	(A) 0.5% aq. FA, (B) ACN (0.5% FA)	0–45 min: 20%–60% B; FR 1.0	Temp. 30°C	MS	[93]
Scutellaria spp.	C_{18} Aquasil, 250 × 4.6 mm, 5 µm	(A) 0.001 M aq. PA, (B) ACN	0–10 min: 20%–30% B; 10–40 min: 30%–35% B; 40–80 min: 35%–65% B; FR 1.0	Temp. 25°C	UV (270)	[158]

Epilobium spp.	C_{18} Hypersil, 250 × 4.6 mm, 5 µm	(A) 2.5% aq. AA, (B) ACN	Glycosides: 0–6 min: 11% B; 6–20 min: 11%–13% B; 20–40 min: 13%–100% B; FR 1.0 Aglycones: 0–20 min: 15% B; 20–21 min: 15%–100% B; 21–30 min: 100% B; FR 1.0	Room temp.	UV (340)	[159]
Hypericum perforatum	C_{18} Luna, 250 × 4.6 mm, 5 µm	(A) 1% aq. triethylamine, pH 4.5 (with AA), (B) ACN	0–40 min: 5%–25% B; 40–60 min: 25%–100% B; 60–80 min: 100% B; FR 1.0		UV, FA, MS, ELSD	[160]
	Discovery HS PEG, 150 × 4.6 mm, 5 µm	(A) 0.1 M aq. AA pH 2.8, (B) MeOH + ACN (5:4 v/v)	0–18 min: 10%–30% B; 18–25 min: 30%–90% B; 25–40 min: 90% B; FR 1.0	Room temp.	UV (270, 590)	[161]
	C_{18} Luna, 250 × 4.6 mm, 5 µm	(A) 25 mM phosphate buffer pH 2.5, (B) MeOH	0–35 min: 5–10% B; 35–50 min: 10–80% B; 50–52 min: 80–100% B	Room temp.	PDA, ESI-MS	[162]
	C_{18} Luna, 150 × 2 mm, 3 µm	(A) 0.1% aq. FA, (B) MeOH				
Dalbergia odorifera	C_{18} Zorbax SB, 250 × 4.6 mm, 5 µm	(A) 0.3% aq. AA, (B) ACN	0–18 min: 25% B; 18–55 min: 25%–46% B; 55–60 min: 46%–80% B; FR 0.8	Temp. 35°C	UV (275)	[163]
Onion, soybeans	C_{18} Hypersil, 125 × 4.6 mm, 3 µm	(A) ACN:water:FA (10:90:5 v/v/v), (B) ACN:water:FA (90:10:5 v/v/v)	0–3 min: 0% B; 3–4 min: 0%–17% B; 4–22 min: 17%–28% B; 22–23 min: 28%–50% B; 23–29.5 min: 50% B; FR 0.9	Room temp.	UV, ESI-MS	[164]
Citrus paradisi *Citrus aurantium* *Citrus sinensis* *Citrus reticulata*	C_{18} Luna, 150 × 2.1 mm, 5 µm	(A) 0.1% aq. FA, (B) ACN (0.1% FA)	0–35 min: 6%–50% B; 35–45 min: 50%–100% B; FR 0.4	Temp. 40°C	PDA	[165]
Gossypium hirsutum *Gossypium herbaceum*	C_{18} YMC Pack Pro, 150 × 4.6 mm, 3 µm	(A) 0.2% aq. PA, (B) MeOH, (C) ACN	A-B-C 90:5:5 (v/v) to A-B-C 70:14:16 (v/v) in 32 min, to A-B-C 45:55:0 (v/v) in 18 min, to A-B-C 0:100:0 (v/v) in 5 min; FR 1.0	Temp. 35°C	UV (360)	[166]

(*Continued*)

TABLE 10.1 (CONTINUED)
Applications of HPLC Gradient Development Methods in Phytochemical Analysis

Compounds and Plant Material	Column	Eluents	Gradient Program	Other Conditions	Detection	Ref.
Morinda citrifolia	C_{18} XTerra, 250 × 4.6 mm, 5 µm	(A) MeOH (0.3% AA), (B) 0.3% aq. AA	0–10 min: 35%–50% A; 10–15 min: 50%–60% A; 15–18 min: 60%–65% A; 18–20 min: 65%–98% A; 20–30 min: 100% A; FR 1.0	Temp. 25°C	PDA, ESI-MS	[167]
Phyllostachys nigra	C_{18} Luna, 250 × 4.6 mm, 5 µm	(A) ACN, (B) 1% aq. AA + NaOH pH 3.0	0–15 min: 15% A; 15–25 min: 15%–40% A; 25–34 min: 40% A; 34–40 min: 40%–15% A; FR 1.0	Temp. 40°C	PDA	[168]
Salvia plebeia	C_{18} Zorbax XDB, 250 × 4.6 mm, 5 µm	(A) 0.5% aq. AA, (B) MeOH	0–14 min: 38%–42% B; 14–17 min: 42%–45% B; 17–17.1 min: 45%–48% B; 17.1–32 min: 48%–50% B; 32–40 min: 50%–85% B; FR 1.0	Temp. 30°C	PDA	[140]
Allium cepa	C_{18} Prevail, 150 × 2.1 mm, 3 µm	(A) 0.1% aq. FA, (B) MeOH (0.1% FA)	0–10 min: 20%–80% B; 10–20 min: 80% B; 20–20.5 min: 80%–20% B; 20.5–25 min: 20% B; FR 0.2	Temp. 30°C	PDA	[169]
	C_{18} Hypersil, 150 × 2.1 mm, 3 µm	(A) 0.1% aq. FA, (B) MeOH (0.1% FA)	0–5 min: 5% B; 5–25 min: 5%–80% B; 25–30 min: 80% B; 30–31 min: 80%–5% B; 31–35 min: 5% B; FR 0.2			
	C_{18} BondaPak, 300 × 3.9 mm	(A) Water:THF:TFA (98:1.9:0.1 v/v), (B) ACN	0–2 min: 17% B; 2–22 min: 17%–90% B; 22–23 min: 90%–95% B; 23–25 min: 95% B; 25–27 min: 95%–17% B; 27–42 min: 17% B; FR 1.0		PDA	[170]
Maytenus ilicifolia *Maytenus aquifolium*	C_{18} Symmetry, 250 × 4.6 mm, 5 µm	(A) 0.5% aq. FA, (B) ACN + MeOH (1:1) + 0.5% FA	0–20 min: 25%–40% B; 20–25 min: 40%–50% B; 25–30 min: 50%–100% B; 30–35 min: 100% B; FR 1.0	Temp. 40°C	PDA, ESI-MS	[171]
Lycium barbarum	C_{18} Luna, 250 × 4.6 mm, 5 µm	(A) 1% aq. AA, (B) ACN	0–10 min: 10% B; 10–30 min: 20% B; 30–60 min: 30% B; 60–90 min: 40% B; 90–120 min: 60% B		UV (370), ESI-MS	[172]

Agrimonia eupatoria	C_{18} Luna, 250 × 21.2 mm, 10 µm	(A) Water, (B) MeOH	0–30 min: 30%–100% B; 30–40 min: 100% B; FR 20		PDA	[173]
Isoflavonoids						
Pueraria thomsonii *Pueraria lobata*	C_{18}, 250 × 4.6 mm, 5 µm	(A) 0.2% aq. AA, (B) ACN	0–10 min: 15% B; 10–12.5 min: 15%–30% B; 12.5–22.5 min: 50% B; 22.5–25 min: 30%–15% B; FR 1.0		PDA	[174]
Soybeans	C_{18} LiChrosphere 100, 250 × 4 mm	(A) 0.1% aq. AA, (B) MeOH (0.1% AA)	0–10 min: 15%–30% B; 10–20 min: 30% B; 20–25 min: 30%–100% B; 25–30 min: 100% B; FR 0.5		PDA, MS	[175]
	YMC ODS-AM, 250 × 3 mm, 5 µm, 150 × 3 mm, 3 µm	(A) 0.1% aq. AA, (B) ACN (0.1% AA)	I. 0–60 min: 10%–30% B II. 0–5 min: 15% B; 5–36 min: 15%–29% B; 36–44 min: 29%–35% B; FR 0.65	Temp. 40°C	UV, MS	[176]
	Chromolith RP-18e	(A) 0.1% AA, (B) MeOH (0.1% AA)	0–2 min: 0%–31% B; 2–4 min: 31% B; 4–5 min: 31%–35% B; 5–8 min: 35% B; 8–9.5 min: 35%–100% B; FR 5.0	Temp. 35°C	PDA	[177]
Soybeans *Trifolium pratense* *Iresine herbstii* *Ononis spinosa*	C_{18} Zorbax SB, 30 × 2.1 mm, 1.8 µm	(A) 0.2% aq. AA, (B) MeOH	0 min: 22% B; 1 min: 80% B; 1.4 min: 100% B; 1.8 min: 22% B; FR 1.4	Temp. 80°C	PDA, ESI-MS	[178]
Ophiopogon japonicus	C_{18} Zorbax 250 × 4.6 mm, 5 µm	(A) 0.3% aq. AA, (B) ACN	0–10 min: 40% B; 10–35 min: 35%–60% B; 35–45 min: 60% B; FR 1.0	Temp. 25°C	PDA, ESI-MS	[179]
Ophiopogon japonicus *Liriope spiccata* *Liriope muscari*	C_{18} Zorbax 250 × 4.6 mm, 5 µm	(A) 0.3% aq. AA, (B) ACN	0–6 min: 25% B; 6–15 min: 25%–95% B; 15–20 min: 95% B; FR 1.0	Temp. 25°C	PDA	[180]
Various plants	C_{18} Alltima, 250 × 4.6 mm, 5 µm	(A) Water (+ 10 mM TFA), (B) ACN (+ 10 mM TFA)	0–30 min: 20%–85% B; FR 1.0		MS, MS/MS	[181]
Acca sellowiana *Psidium guajava* *Psidium littorale*	C_8 Zorbax XDB, 150 × 4.6 mm, 5 µm	(A) 0.3% aq. FA, (B) ACN	0–20 min: 15%–35% B; 20–25 min: 35%–50% B; 25–30 min: 50%–55% B; 30–45 min: 55%–85% B; FR 0.8	Temp. 40°C	PDA, MS	[182]

(*Continued*)

TABLE 10.1 (CONTINUED)
Applications of HPLC Gradient Development Methods in Phytochemical Analysis

Compounds and Plant Material	Column	Eluents	Gradient Program	Other Conditions	Detection	Ref.
Trifolium pratense *Glycine max* *Pisum sativum* *Ononis spinosa*	Various	(A) 0.3% aq. AA, (B) MeOH	Various	Various	PDA	[183]
Anthocyanins						
Echium plantagineum	C_{18}, 150 × 4.6 mm, 5 µm	(A) 0.1% aq. TFA, (B) ACN	0–5 min: 10% B; 5–20 min: 10%–13% B; 20–35 min: 13%–15% B; 35–55 min: 15%–35% B; 55–65: 35%–10% B; FR 0.5	Temp. 35°C	PDA, MS	[184]
Rubus idaeus	C_{18} Kromasil, 250 × 4.6 mm, 5 µm	(A) 3% aq. FA, (B) FA:water:MeOH (3:47:50 v/v)	0–2 min: 40% B; 2–42 min: 40%–100% B; FR 0.8		UV (520), ESI-MS	[185]
Various						
Paeonia lactiflora *Ligusticum chuanxiong*	C_{18} Hypercil, 250 × 4 mm, 5 µm	(A) 0.1% aq. AA, (B) ACN (0.1% AA)	0–5 min: 0% B; 5–10 min: 0%–2.5% B; 10–15 min: 2.5% B; 15–35 min: 2.5%–10% B; 35–55 min: 10%–25% B; 55–80 min: 25%–55% B; FR 0.8	Temp. 30°C	PDA, ESI-MS	[186]
Rhodiola heterodonta *Rhodiola semenovii* *Rhodiola rosea*	C_{18} VYDAC, 150 × 2.1 mm	(A) 5% aq. ACN (+ 0.1% FA), (B) 95% aq. ACN (+ 0.1% FA)	Step gradient: 0%, 30%, 60%, 90%, 0% B at 3, 30, 45, 50, and 60 min, respectively; FR 0.2		ESI-MS	[187]
Echinacea pallida	C_{18} LiChrospher, 125 × 4 mm, 5 µm Chromolith RP-18e, 100 × 4.6 mm	(A) Water, (B) ACN	I. 0–30 min: 40%–80% B; FR 1.0 II. 0–20 min: 40%–80% B; FR 2.0	Temp. 20°C	PDA	[188]
Pseudostellaria heterophylla	C_{18} ODS-BP, 200 × 4.6 mm, 5 µm	(A) Water, (B) ACN	0–10 min: 2%–10% B; 10–30 min: 10%–45% B; 30–40 min: 45%–55% B; 40–60 min: 55%–90% B; 60–65 min: 90%–100% B; 65–75 min: 100% B; FR 1.0	Temp. 30°C	PDA	[189]

Ligusticum chuanxiong *Angelica sinensis*	C_{18} Cosmosil, 250 × 4.6 mm, 5 µm	(A) 20 mM KH_2PO_4 pH 3.5, (B) ACN:water (80:20 v/v)	0 min: 0% B; 20 min: 10% B; 30 min: 12% B; 40 min: 30% B; 45 min: 60% B; 50 min: 100% B; 55 min: 100% B; 60 min: 0% B; FR 0.8		PDA, ESI-MS, APCI-MS	[190]
Daucus carota	C_{18} Luna, 150 × 4.6 mm, 3 µm	(A) Water, (B) ACN	0–5 min: 20% B; 5–10 min: 20%–50% B; 10–30 min: 50%–53% B; 30–45 min: 53%–65% B; 45–50 min: 65% B; 50–70 min: 65%–75% B; 70–72 min: 75% B; 72–90 min: 75%–95% B; 90–95 min: 95% B; 95–100 min: 95%–20% B; 100–110 min: 20% B; FR 1.0	Temp. 40°C	PDA	[191]
Herbal preparations	UPLC BEH C_{18}, 100 × 2.1 mm	(A) 0.1% aq. FA, (B) ACN:iPrOH (1:1 v/v) + 0.1% FA	0–4.5 min: 10%–50% B; FR 0.4	Temp. 65°C	MS	[192]
Arabidopsis thaliana *Botrytis cinerea*	C_{18} Gemini, 150 × 2 mm, 5 µm	(A) 0.1% aq. FA, (B) MeOH (0.1% FA)	0–2 min: 30% B; 2–20 min: 30%–100% B; FR 0.3		ESI-MS/MS	[193]
Juglans regia	C_{18} Sinochrom, 250 × 4.6 mm, 5 µm	(A) ACN, (B) 2% aq. AA	0–15 min: 80%–30% B; 15–20 min: 30% B; 20–21 min: 30%–80% B; 21–25 min: 80% B; FR 1.0	Temp. 25°C	PDA	[194]
Ganoderma lucidum	C_{18} Kromasil, 250 × 4.6 mm, 5 µm	(A) 0.1% aq AA, (B) ACN	0–40 min: 30%–32% B; 40–60 min: 32%–40% B; 60–65 min: 40% B; 65–70 min: 40%–82% B; 70–85 min: 82%–100% B; FA 0.8	Temp. 35°C	PDA, ESI-MS	[195]
Angelica sinensis *Astragalus membranaceus*	C_{18} Gemini, 250 × 4.6 mm, 5 µm	(A) 0.3% aq. FA, (B) ACN	0–15 min: 20% B; 15–25 min: 20%–32% B; 25–34 min: 32%–34% B; 34–45 min: 34%–48% B; 45–65 min: 48%–65% B; 65–70 min: 65% B; FR 1.0	Temp. 20°C	PDA, ELSD	[196]
Phenolic acids						
Dactylis glomerata *Festuca rubra* *Festuca ovina* *Bromus marginatus* *Tilia cordata* *Uncaria tormentosa*	C_{18} Hypersil BDS, 100 × 4.6 mm, 3 µm	(A) 0.3% aq. AA, (B) MeOH	0–7 min: 7%–20% B; 7–12 min: 20%–35% B; 12–18 min: 35% B; 18–20 min: 35%–60% B; 20–25 min: 60%–80% B; 25–27 min: 80%–100% B; 27–30 min: 100% B; 30–35 min: 100%–7% B; FR 0.6	Temp. 30°C	PDA	[197]

(*Continued*)

TABLE 10.1 (CONTINUED)
Applications of HPLC Gradient Development Methods in Phytochemical Analysis

Compounds and Plant Material	Column	Eluents	Gradient Program	Other Conditions	Detection	Ref.
Echinacea spp.	C_{18} LiChrospher, 125 × 4 mm, 5 µm	(A) 0.1% aq. PA, (B) ACN	0–13 min: 10%–22% B; 13–18 min: 22%–10% B; 18–21 min: 10% B; FR 1.5	Temp. 26°C	PDA	[198]
Green coffee beans	C_{18} Luna, 150 × 4.6 mm, 5 µm	(A) 0.03% aq. TFA, (B) ACN	0–90 min: 10%–100% B; FR 1.0	Room temp.	UV (330)	[199]
Hippophaë rhamnoides	C_{18} ODS-2, 250 × 4.6 mm, 5 µm	(A) 2% aq. AA, (B) MeOH	0–5 min: 100% A; 5–10 min: 90% A; 10–15 min: 90% A; 15–20 min: 80% A; 20–25 min: 80% A; 25–40 min: 40% A; 40–60 min: 0% A; FR 1.0	Temp. 35°C	PDA	[200]
Eucommia ulmodies	C_{18}, 150 × 4.6 mm, 5 µm	(A) MeOH, (B) 0.5% aq. AA	0–16 min: 30% A; 17–25 min: 30%–60% A; 25–26 min: 60%–30% A; FR 1.0	Temp. 30°C	PDA, APCI-MS	[201]
Phenolics						
Ananas comosus	C_{18} Kromasil, 150 × 4.6 mm, 5 µm	(A) 0.1% aq. FA, (B) ACN (0.1% FA)	0–50 min: 5%–20% B; 50–90 min: 20%–40% B; FR 1.0		PDA, ESI-MS	[202]
Propolis	C_{18} Waters Symmetry, 250 × 4.6 mm, 5 µm	(A) 0.1% aq. FA, (B) ACN	0–6 min: 20% B; 6–10 min: 20%–30% B; 10–40 min: 30%–40% B; 40–60 min: 40%–60% B; 60–80 min: 60%–90% B; 80–90 min: 90% B; FR 1.2	Temp. 30°C	PDA, ESI-MS	[203]
Chrysanthemum indicum	C_{18} Hypersil, 200 × 4.6 mm, 5 µm	(A) 1% aq. AA, (B) ACN	0–4 min: 10% B; 4–15 min: 10%–30% B; 15–25 min: 30% B; 25–30 min: 30%–10% B; FR 1.0	Temp. 25°C	PDA	[204]
Fragaria x ananassa	C_{18} Symmetry, 250 × 4.6 mm, 5 µm	(A) 1% aq. FA, (B) ACN	0–10 min: 10% B; 10–70 min: 10%–20% B; FR 1.0		PDA, ESI-MS	[205]
Rheum tanguticum	C_{18} Kromasil, 250 × 4.6 mm, 5 µm	(A) 0.1% aq. PA, (B) ACN	0–10 min: 4%–11% B; 10–25 min: 11%–13% B; 25–50 min: 13%–15% B; FR 1.0	Temp. 40°C	UV (280)	[206]

Camellia sinensis	C_{18} Lichrocart, 250 × 4 mm, 5 µm	(A) 0.1% aq. PA, (B) ACN	0–3 min: 10% B; 3–10 min: 10%–20% B; 10–12 min: 20%–35% B; 12–15 min: 35% B; 15–18 min: 35%–10% B; FR 1.0	Temp. 35°C	PDA	[207]
Polygonum cuspidatum	SB-C18 Zorbax, 250 × 4.6 mm, 5 µm	(A) 0.1% aq. FA, (B) ACN	0–15 min: 15%–20% B; 15–60 min: 20%–80% B; FR 1.0	Temp. 25°C	PDA	[208]
Phyllanthus niruri	C_{18} LiChrospher, 250 × 4 mm, 5 µm	(A) 1% aq. PA (w/w), (B) ACN + 1% aq. PA (1:1 v/v)	0–7 min: 22%–24% B; 7–17 min: 24%–40% B; 17–25 min: 40%–100% B; 25–40 min: 100%–22% B; FR 0.6		PDA	[209]
Green tea	ODS-HG Develisil, 150 × 4.6 mm	(A) Water:ACN:85% PA (95.45:4.5:0.05 v/v), (B) water:ACN:85% PA (49.95:50.0:0.05 v/v)	0–5 min: 10% B; 5–8 min: 10%–30% B; 8–10 min: 30% B; 10–15 min: 30%–80% B; 15–20 min: 80% B; FR 1.0	Temp. 40°C	PDA	[210]
Ixeris sonchifolia	ODS2 Hypersil, 250 × 4.6 mm, 5 µm	(A) 0.5% aq. AA, (B) ACN	0–13 min: 7%–11% B; 13–30 min: 11%–18% B; 30–45 min: 18%–28% B; 45–55 min: 28% B; FR 1.0	Temp. 30°C	UV (335)	[211]
Smallanthus sonchifolius Beverages, wines, teas	Various	Various	Various	Temp. 36°C	coulometric	[212]
Vanilla planifolia	Purospher-Star RP-18e, 250 × 4.6 mm, 5 µm	(A) 0.2% aq. AA, (B) ACN:MeOH (1:1 v/v)	0.01–8 min: 10%–30% B (FR 1.5); 8–15 min: 30%–50% B (FR 1.5–1.0); 15–20 min: 50%–70% B (FR 1.0–0.8); 20–25 min: 70%–80% B (FR 0.8–0.6)	Temp. 25°C	PDA	[213]
Rosa damascena *Rosa brunonii* *Rosa bourboniana*	C_{18} Luna, 250 × 4.6 mm, 5 µm	(A) Water, (B) ACN (0.02% TFA)	5 min: 80% A; 8 min: 20%–40% A; 12 min: 40%–50% A; 17 min: 50%–40% A; 21 min: 40%–20% A; FR 1.0	Temp. 27°C	PDA	[214]
Salix alba *Salix daphnoides* *Salix herbacea* *Salix viminalis* *Salix purpurea* *Salix triandra*	Various	Various	Various	Various	UV, ELSD	[215]
Myrciaria spp. *Eugenia* spp. *Syzygium* spp.	C_{18} Aqua, 250 × 4.6 mm, 5 µm	(A) 1% aq. FA, (B) ACN	0–30 min: 90%–75% A; 30–45 min: 75%–40% A; FR 1.0		PDA	[216]

(*Continued*)

TABLE 10.1 (CONTINUED)
Applications of HPLC Gradient Development Methods in Phytochemical Analysis

Compounds and Plant Material	Column	Eluents	Gradient Program	Other Conditions	Detection	Ref.
Yucca gloriosa	C_{18} Symmetry, 150 × 2.1 mm, 5 µm	(A) 0.05% aq. TFA, (B) ACN (0.05% TFA)	0–30 min: 20%–30% B; 30–40 min: 30% B; 40–70 min: 30%–60% B; FR 0.3		ESI-MS	[217]
Epimedium spp.	C_{18} Zorbax SB, 250 × 4.6 mm, 5 µm	(A) ACN, (B) 0.1% aq. FA	0–15 min: 12%–25% A; 15–30 min: 25% A; 30–55 min: 25%–35% A; 55–70 min: 35%–60% A; 70–80 min: 60%–90% A; FR 1.0	Temp. 40°C	PDA, ESI-MS	[218]
Argania spinosa	C_{18} Luna, 50 × 2.1 mm, 3.5 µm	(A) 0.05% aq. AA, (B) ACN	0 min: 94% A; 5 min: 83.5% A; 7 min: 82.5% A; 12.5 min: 81.5% A; 21 min: 0% A; 23 min: 94% A; FR 0.6		UV(280), ESI-MS	[219]
Cynara scolimus	C_{18} Luna, 50 × 2.1 mm, 3.5 µm	(A) 0.1% aq. FA, (B) ACN (0.1% FA)	0 min: 94% A; 5 min: 83.5% A; 7 min: 82.5% A; 12.5 min: 81.5% A; 21 min: 0% A; 23 min: 94% A; FR 0.4		PDA, MS	[220]
Hamamelis virginiana	C_{18} Kingsorb, 150 × 4.6 mm, 5 µm	(A) 0.1% aq. PA, (B) MeOH (0.1% PA)	0–15 min: 21% B; 15–20 min: 21%–50% B; FR 1.0	Temp. 30°C	UV (210)	[221]
Rhus coriaria	Hypersil R-Osil, 250 × 4.6 mm, 5 µm	(A) Hexane, (B) MeOH:THF (3:1 v/v)	0–15 min: 10%–50% B; 15–30 min: 50%–65% B; FR 1.0	Temp. 25°C	PDA	[222]
Chironia krebsii *Halenia corniculata* *Gentiana ottonis* *Swertia calycina*	C_{18} Nova-Pak, 150 × 3.9 mm, 4 µm	(A) 0.05% aq. TFA, (B) ACN (0.05% TFA)	0–50 min: 5%–65% B; FR 1.0		PDA, MS, NMR	[223]
Saponins						
Salvia miltiorrhizae *Panax notoginseng*	C_{18} Zorbax, 250 × 4.6 mm, 5 µm	(A) 0.1% aq. AA, (B) ACN (0.1% AA)	0–15 min: 20%–35% B; 15–25 min: 35% B; 25–40 min: 35%–43% B; 40–50 min: 43%–46% B; 50–65 min: 46%–58% B; 65–70 min: 58%–75% B; FR 0.8	Temp. 30°C	PDA, ESI-MS	[224]

Symplocos chinensis	C_{18} YMC Pack Pro, 150 × 3.0 mm, 5 µm	(A) 0.1% aq. FA, (B) MeOH	0–5 min: 80%–85% B; 5–20 min: 85% B; 20–25 min: 85%–95% B; 25–30 min: 95%–80% B; FR 0.3		ESI-MS	[225]
Medicago sativa *Medicago truncatula*	C_{18}, 250 × 4.6 mm, 5 µm	(A) 1% aq. AA, (B) ACN	0–60 min: 20%–80% B; FR 0.8		PDA, ESI-MS	[226]
Bacopa monniera	C_{18} LiChrospher, 125 × 5 mm, 5 µm	(A) D_2O, (B) MeOH	0–30 min: 60%–90% B; FR 0.8		UV, NMR, APCI-MS	[227]
Stephanotis mucronata	C_{18} Extend, 150 × 4.6 mm, 3.5 µm	(A) Water, (B) MeOH	0–10 min: 60% B; 10–20 min: 60%–65% B; 20–40 min: 65%–85% B; FR 0.8	Room temp.	UV (210), ESI-MS, NMR	[228]
Panax notoginseng	C_{18} Zorbax SB, 250 × 4.6 mm, 5 µm	(A) Water, (B) ACN	0–30 min: 18%–19% B; 30–40 min: 19%–31% B; 40–60 min: 31%–56% B; FR 1.5	Temp. 40°C	UV (203)	[229]
Swartzia madagascariensis *Phytolacca dodecandra*	C_{18} Nova-Pak, 150 × 3.9 mm, 4 µm	(A) 0.05% aq. TFA, (B) ACN (0.05% TFA)	0–30 min: 30%–50% B; FR 0.9		PDA, MS, NMR	[223]
Ginsenosides						
Various samples	VP-ODS, 150 × 2 mm, 5 µm	(A) ACN:water (10:90 v/v), (B) ACN:water (45:55 v/v)	0–21 min: 30% B; 21–21.1 min: 30%–57% B; 21.1–51 min: 57% B; 51–51.1 min: 57%–64% B; 51.1–70 min: 64% B; FR 0.22	Temp. 25°C	UV	[230]
Ginkgo biloba, food samples	C_{18} Hyperclone, 150 × 3.2 mm, 5 µm	(A) Water, (B) MeOH	0–1 min: 30% B; 1–7 min: 30%–45% B; 7–10 min: 45% B; 10–12 min: 45%–70% B; 12–13 min: 70% B; 13–14 min: 70%–30% B; 14–15 min: 30% B; FR 0.55	Room temp.	APCI-MS	[231]
Panax quinquefolium	C_{18} Spherisorb, 250 × 4.6 mm, 5 µm	(A) Water, (B) ACN	0–20 min: 25%–30% B; 20–21 min: 30%–90% B; 21–30 min: 90% B; 30–31 min: 90%–25% B; 31–40 min: 25% B; FR 1.6	Room temp.	PDA, ELSD	[232]
	C_{18} Hypersil, 250 × 4.6 mm, 5 µm	(A) Water, (B) ACN	0–15 min: 10%–40% B; 15–25 min: 40%–100% B; FR 1.0	Temp. 40°C	PDA	[233]

(*Continued*)

TABLE 10.1 (CONTINUED)
Applications of HPLC Gradient Development Methods in Phytochemical Analysis

Compounds and Plant Material	Column	Eluents	Gradient Program	Other Conditions	Detection	Ref.
Panax ginseng	C_{18} Zorbax Eclipse XDB, 250 × 4.6 mm, 5 µm	(A) ACN, (B) water	0–30 min: 20% A; 30–40 min: 20%–31% A; 40–60 min: 31%–39% A; 60–70 min: 39%–50% A; 70–85 min: 50%–85% A; FR 1.0	Temp. 25°C	PDA, ESI-MS	[234]
	C_{18} Pinnacle, 250 × 4.6 mm, 5 µm	(A) Water, (B) ACN	0–30 min: 19% A; 30–31 min: 19%–30% B; 31–65 min: 30% B; FR 1.5	Temp. 25°C	UV (203)	[235]
Terpenoids						
Kadsura heteroclita	C_{18} Eclipse XDB, 250 × 4.6 mm, 5 µm	(A) 0.5% aq. PA, (B) ACN	0–30 min: 60%–100% B; 30–35 min: 100% B; FR 1.0	Temp. 30°C	UV (210)	[236]
Switenia macrophylla	Phenyl-hexyl, 250 × 4.6 mm, 5 µm	(A) D_2O, (B) ACN	0–30 min: 30%–90% B; FR 1.0	Temp. 35°C	UV, NMR	[237]
Stevia rebaudiana	C_{18} Biospher, 250 × 4 mm, 7 µm	(A) Water, (B) ACN	0–30 min: 15%–50% B; FR 1.0	Temp. 25°C	PDA	[238]
Tripterygium wilfordii	C_8 XDB Zorbax, 250 × 4.6 mm, 5 µm	(A) 0.05% aq. FA, (B) ACN (0.05% FA)	0–5 min: 35%–40% B; 5–30 min: 40%–50% B; 30–50 min: 50%–60% B; 50–65 min: 60%–95% B; FR 0.8	Temp. 30°C	ELSD	[239]
Achillea millefolium *Achillea collina* *Achillea pratensis*	C_{18} Kovasil MS, 33 × 2 mm, 1.5 µm	(A) ACN:water (2:98 v/v) + 1 mmol CH_3COONH_4, (B) ACN:water (95:5 v/v) + 1 mmol CH_3COONH_4	0–20 min: 0%–32% B; 20–20.1 min: 32%–100% B; 20.1–25 min: 100% B; 25–25.5 min: 100%–0% B; 25.5–30 min: 0% B; FR 0.2		PDA, APCI-MS	[240]
Salvia miltiorrhiza	C_{18} LiChrospher, 250 × 4.6 mm, 5 µm	(A) 0.1 aq. FA, (B) ACN	0–10 min: 10%–20% B; 10–27 min: 20%–33% B; 27–30 min: 33%–70% B; 30–50 min: 70%–85% B; FR 1.0	Temp. 20°C	UV (280)	[241]

Coumarins						
Dendrobium thyrsiflorum	C_{18} Polaris, 250 × 4.6 mm, 5 µm	(A) 0.5% aq. AA, (B) ACN	0–20 min: 10%–20% B (FR 0.8); 20–35 min: 20%–30% B (FR 1.0); 34–45 min: 30%–90% B (FR 1.0)	Temp. 40°C	PDA, ESI-MS	[242]
Ammi majus *Ruta graveolens*	C_{18} LiChrospher, 250 × 4 mm, 5 µm	(A) Water, (B) MeOH, (C) THF	0–20 min: 5%–13% B; 20–52 min: 13%–20% B, 5%–7% C; 52–59 min: 65% B; FR 1.5	Temp. 30°C	PDA	[243]
Alkaloids						
Tinospora sagittata *Tinospora capillipes*	C_{18} YDC, 250 × 4.6 mm, 5 µm	(A) Buffer (0.02 M NaH_2PO_4 + 0.01 M triethylamine + PA, pH 3), (B) ACN	0–5 min: 20%–30% B; 5–14 min: 30%–39% B; FR 1.0	Temp. 30°C	PDA	[244]
Aconitum spp.	C_{18} Alltima, 250 × 4.6 mm, 5 µm	(A) Buffer (10 mM ammonium bicarbonate + ammonia to pH 10), (B) ACN	0–10 min: 20%–25% B; 10–30 min: 25%–34% B; 30–67 min: 34%–45% B; 67–75 min: 45%–60% B; FR 1.0		PDA	[245]
Corydalis solida *Corydalis cava* *Corydalis pumila* *Corydalis intermedia*	C_{18} Max RP, 150 × 4.6 mm, 4 µm	(A) 10 mM ammonium acetate buffer pH 5.9, (B) ACN	DAD-MS/MS: 0–25 min: 15%–40% B; 25–35 min: 40%–60% B; 35–45 min: 60%–98% B; FR 1.0 SPE-NMR: as above, FR 0.8	Temp. 15°C Room temp.	PDA, ESI-MS NMR	[246]
Veratrum dahuricum	C_{18} Zorbax, 250 × 4.6 mm, 5 µm	(A) 0.1 aq. FA + 0.04% ammonia, (B) ACN	0–6 min: 5%–20% B; 6–40 min: 20%–35% B; 40–45 min: 35%–100% B; FR 0.7	Temp. 25°C	ESI-MS, ELSD	[247]
Fritillaria spp.	C_{18} Extend Zorbax, 150 × 4.6 mm, 5 µm	(A) 0.03% aq. diethylamine, (B) ACN (0.03% diethylamine)	0–15 min: 30%–40% B; 15–35 min: 40%–45% B; 35–50 min: 45%–65% B; 50–60 min: 65% B; 60–75 min: 65%–80% B; 75–80 min: 80%–100% B; 80–95 min: 100% B; FR 0.8	Temp. 25°C	ESI-MS	[248]
Corydalis yanhusuo	C_{18} Diamonsil, 200 × 4.6 mm, 5 µm	(A) 0.2% aq. AA (+ triethylamine, pH 5), (B) ACN	0–15 min: 20% B; 15–35 min: 20%–80% B; 35–37 min: 80%–20% B; FR 1.0	Temp. 25°C	PDA, ESI-MS	[249]

(*Continued*)

TABLE 10.1 (CONTINUED)
Applications of HPLC Gradient Development Methods in Phytochemical Analysis

Compounds and Plant Material	Column	Eluents	Gradient Program	Other Conditions	Detection	Ref.
Steroids						
Vitex polygama	Phenyl-hexyl, 250 × 4.6 mm, 5 µm Phenyl-hexyl, 150 × 4.6 mm, 10 µm	(A) Water, (B) MeOH	0–30 min: 5%–100% B; FR 1.0 0–21 min: 35%–100% B; FR 1.0	Room temp.	PDA	[250]
Iochroma gesnerioides	C_{18} Nucleosil, 125 × 4 mm, 5 µm	(A) 5% aq. ACN, (B) 95% aq. ACN	0–10 min: 27% B; 10–20 min: 27%–35% B; 20–45 min: 35% B; FR 1.0	Room temp.	UV (215)	[251]
Antraquinones						
Polygonum cuspidatum	VP-ODS, 250 × 4.6 mm, 5 µm	(A) Water, (B) MeOH	0–6 min: 45% B; 6–9 min: 45%–75% B; 9–18 min: 75%–100% B; 18–25 min: 100% B; FR 1.0	Temp. 25°C	PDA	[252]
Rubia tinctorum	C_{18} Alltima, 250 × 3.2 mm, 5 µm	(A) Water, (B) ACN	0–6 min: 27% B; 6–20 min: 27%–70% B; 20–35 min: 70% B; 34–40 min: 70%–27% B; 40–45 min: 27% B; FR 1.0	Room temp.	PDA	[253]
Antioxidants						
Olea europaea	Spherisorb ODS-2, 250 × 4.6 mm, 5 µm	(A) 0.2% aq. AA, (B) MeOH	0–5 min: 10%–20% B; 5–10 min: 20% B; 10–40 min: 20%–80% B; 40–45 min: 80% B; 45–50 min: 80%–10% B	Room temp.	PDA	[254]
Tilia europea *Urtica dioica* *Lonicera periclymenum* *Hypericum perforatum*	C_{18} Altima, 150 × 4.6 mm, 5 µm	(A) 0.1% aq. FA, (B) ACN	0–5 min: 10% B; 5–8 min: 10%–30% B; 8–18 min: 30%–70% B; 18–22 min: 70%–100% B; 22–27 min: 100% B; 27–30 min: 100%–10% B; FR 0.6		MS, UV, NMR	[255]
Lignans						
Piper longum	C_{18} Discovery HS F5, 250 × 4.6 mm, 5 µm	(A) Water, (B) ACN	0–20 min: 50%–70% B; 20–40 min: 70% B; 40–50 min: 70%–50% B; FR 1.0		PDA	[256]

Torreya jackii	C_{18} LiChrospher, 250 × 4 mm	(A) 0.1% TFA in D_2O, (B) ACN	0–30 min: 25%–40% B; 30–40 min: 40%–55% B; FR 0.8		UV, NMR	[257]
Herpetospermum caudigerum	C_{18} DB, 250 × 4.6 mm, 5 µm	(A) ACN, (B) 0.1% aq. PA	0–30 min: 21% A; 30–55 min: 21–29% A; 55–65 min: 29% A	Temp. 25°C	UV (240)	[258]
Carotenoids						
Arabidopsis thaliana	YMC30, 100 × 2.1 mm, 3 µm	(A) 3% water in MeOH, (B) TBME	0–12 min: 3% B; 12–13 min: 3%–38% B; 13–15 min: 38% B; 15–16 min: 38%–68%B; 16–21 min: 68% B; 21–25 min: 68%–3% B; FR 0.5	Temp. 25°C	PDA, MS	[64]
Rubus idaeus *Rubus fruticosus* *Fragaria vesca* *Vaccinium myrtillus* *Ribes nigrum* *Ribes rubrum*	C_{18} Prevail, 150 × 4.6 mm, 5 µm	(A) ACN:MeOH (95:5 v/v), (B) ACN:MeOH:AcOEt (60:20:20 v/v)	0–5 min: 0% B; 5–13 min: 0%–100% B; FR 1.0	Temp. 28°C	UV (450)	[259]
Iridoids						
Gentiana manshurica *Gentiana scabra* *Gentiana triflora* *Gentiana rigescens*	C_{18} Beckman, 250 × 4.6 mm, 5 µm	(A) Water, (B) ACN	0–22.5 min: 10% B; 22.5–25 min: 10%–20% B; 25–32.5 min: 20% B; 32.5–35 min: 20%–10% B		PDA	[260]
Amino acids, amines						
Centaurea solstitialis	C_{18} Kromasil, 250 × 4.6 mm, 5 µm	(A) Phosphate buffer pH 7.0, (B) ACN	0–15 min: 13% B; 15–40 min: 13%–50% B; 40–60 min: 50%–85% B; 60–62 min: 85% B; FR 0.8	Temp. 30°C	UV, SFD	[261]
Taxoids						
Taxus baccata var. *Elegantissima* *Taxus baccata* var. *Aurea*	C_{18} Symmetry, 150 × 4.6 mm, 3.5 µm	(A) Water, (B) ACN	0–8 min: 25% B; 8–16 min: 25%–44% B; 16–20 min: 44% B; 20–45 min: 44%–80% B; 45–50 min: 80%–25% B; FR 0.75	Temp. 25°C	PDA	[262]

(*Continued*)

TABLE 10.1 (CONTINUED)
Applications of HPLC Gradient Development Methods in Phytochemical Analysis

Compounds and Plant Material	Column	Eluents	Gradient Program	Other Conditions	Detection	Ref.
Taxus wallichiana	C_{18} Nova-Pak, 150 × 3.9 mm, 4 µm C_{18} Symmetry, 150 × 3.9 mm, 5 µm Nova-Pak Phenyl, 4 µm Curosil-B, 250 × 4.6 mm, 3 µm	(A) MeOH:ACN:water (20:5:75 v/v/v), (B) MeOH:ACN:water (20:45:35 v/v/v) or MeOH:ACN:water (30:35:35 v/v/v)	I. 0–15 min: 80% A (FR 0.8); 15–20 min: 80%–60% A (FR 0.8); 20–30 min: 60%–40% A (FR 1.0); 30–40 min: 40%–0% A (FR 1.0); 40–50 min: 0% A (FR 1.0); 50–55 min: 0%–80% A (FR 1.0), 55–65 min: 80% A (FR 0.8) II. Concave (profile 3) III. 0–20 min: 80%–60% A (FR 1.0); 20–26 min: 60%–47% A (FR 1.0); 26–31 min: 47%–20% A (FR 1.0); 31–37 min: 20%–0% A (FR 1.0); 37–45 min: 0% A (FR 1.2)	Temp. 27°C	PDA	[263]
Taxus spp.	Ultracarb ODS5, 150 × 4.6 mm, 5 µm	(A) 0.05 M CH_3COONH_4: ACN (7:3 v/v), (B) 0.05 M CH_3COONH_4:ACN (1:9 v/v)	0–30 min: 0%–34% B; 30–32 min: 34%–0% B; FR 0.8		PDA	[264]
Taxus cuspidata *Taxuschinensis* *Taxus media*	C_{18} HIQ SIL, 250 × 4.6 mm, 5 µm	(A) Water, (B) ACN	0–16 min: 28% B; 16–17 min: 28%–33% B; 17–46 min: 33% B; 46–60 min: 33%–45% B; FR 1.0	Temp. 25°C	PDA	[265]
Fatty acids						
Gentiana straminea *Gentiana dahurica*	C_8 XDB Eclipse, 150 × 4.6 mm, 5 µm	(A) ACN:water (1:1 v/v), (B) ACN:water + 0.2 M $HCOONH_4$ buffer, (C) ACN:DMF (100:2 v/v), (D) ACN:DMF (100:30 v/v)	0 min: 95% A, 5% C; 4 min: 95% A, 5% C; 4.2 min: 95% B, 5% C; 8 min: 95% B, 5% C; 8.5 min: 75% B, 25% C; 15 min: 50% B, 50% C; 40 min: 100% C; 48 min: 100% D; 65 min: 100% D; FR 1.0	Temp. 30°C	FLU, APCI-MS	[266]

Note: AA, acetic acid; ACN, acetonitrile; AcOEt, ethyl acetate; APCI, atmospheric pressure chemical ionization; DMF, dimethylfuran; ELSD, evaporative light scattering detection; ESI, electrospray ionization; FA, formic acid; FLU, fluorescence; FR, flow rate (in mL/min); iPrOH, isopropyl alcohol; MeOH, methanol; MS, mass spectrometry; NMR, nuclear magnetic resonance; PA, phosphoric acid; PDA, photodiode array detection; SFD, spectrofluorimetric detection; TFA, trifluoroacetic acid; THF, tetrahydrofuran; UV, ultraviolet (wavelengths given in nm).

hydrophilic. Although RP-HPLC has been the primary separation means for the procyanidins, past studies illustrates the difficulty in determining the degree of polymerization of these antioxidants. Hammerstone et al. therefore have developed a normal-phase HPLC method that utilizes a series of linear gradients of methanol into dichloromethane with a constant amount of acetic acid and water [51]. The separation of some flavonoids from various groups is presented in Figure 10.5.

The combination of mass spectrometry with various chromatographic techniques (including gradient HPLC methods) has proved to be highly succesfull in the examination of the chemical nature of phenolics in biological matrices. The coupling of liquid chromatography and mass spectrometry is now the one of the best methods for analyzing and identifying naturally occurring compounds [52]. For the detailed separation method for polyphenols, readers are referred to a recent book by Santos-Buelga and Williamson [53] and reviews [54,55].

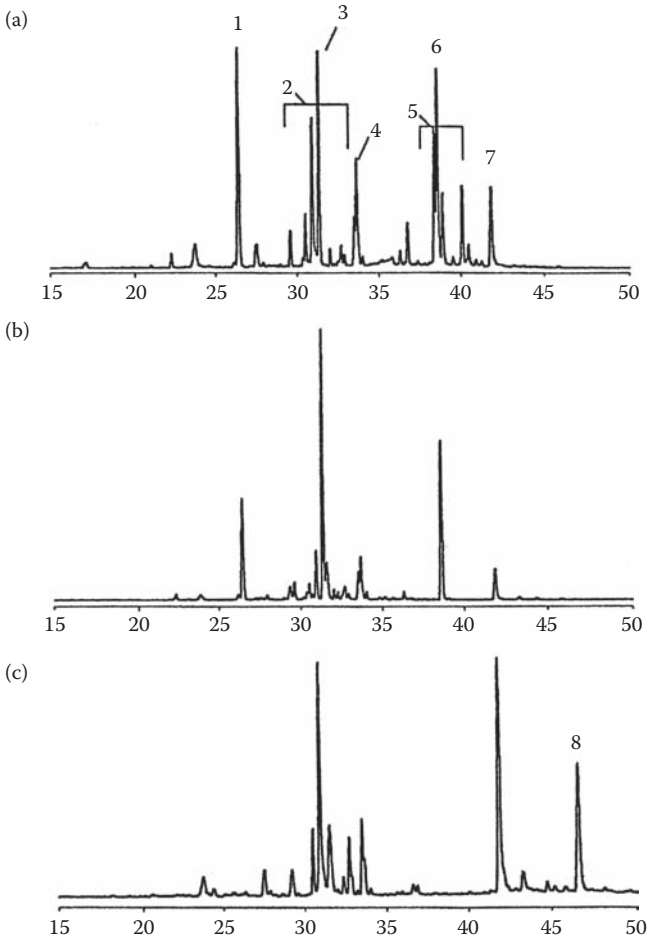

FIGURE 10.5 HPLC chromatograms (absorbance at 352 nm vs. time [min]) of (a) a flavonoid mixture showing seven different groups of compounds: (1) a kaempferol triglycoside, (2) a set of apigenin glycosides, (3) a kaempferol diglycoside, (4) a luteolin glycoside, (5) a set of acylated kaempferol glycosides, (6) a chalcone, and (7) luteolin; (b) the alkaline hydrolysis product of the same mixture, showing a large relative increase in peak 3 and loss of the acylated kaempferol glycoside peaks; and (c) the acid-hydrolyzed mixture showing luteolin and kaempferol. Peaks due to apigenin glycosides are still present, showing these are apigenin *C*-glycosides. (Reprinted from Robards K., *J. Chromatogr. A*, 1000, 657–691, 2003. With permission.)

10.5.2 Carbohydrates

The separations of glycoproteins and glycopeptides are generally performed by gradient elution with increasing organic-solvent concentration in the eluent. Also, most derivatized saccharides are better separated by gradient elution. For reversed-phase liquid chromatography of saccharides, the mobile-phase composition must be selected to provide an adequate resolution and rapid analysis time. For reversed-phase liquid chromatography of proteins and glycoproteins, in addition to achieving high resolution and rapid analysis, the composition of the mobile phase must be adequately selected to minimize protein denaturation during the chromatographic run and to maximize protein recovery from the column. The pH, the nature of the organic solvent, and other additives all have an effect on the quality of protein separation and purification.

Very recently, native and reduced α-glucan oligosaccharides were successfully separated on a C_{18} column for up to a pore diameter (dp) > 40 using a gradient elution with acetonitrile and a postcolumn reactor with co-immobilized glucoamylase and glucose oxidase for the detection after RPLC separation [56].

The retention behavior of oligosaccharide alditols was evaluated on a Hypercarb PGC column [57]. The oligosaccharides with a dp between 1 and 6 were substantially retained and could be eluted in a gradient from 0 to 25% acetonitrile (v/v) in 0.05% aqueous trifluoroacetic acid. In this study, the separation of oligosaccharide alditol isomers has shown that PGC is a useful adjunct to C_{18}-silica stationary phases. However, fucosylated pentasaccharide isomers, which were successfully separated using a C_{18}-silica column, were not resolved with the PGC column.

More examples of carbohydrate separation methods are described by Churms [58].

10.5.3 Carotenoids

The lipophilic characteristics of carotenoids have made normal-phase HPLC a more favorable choice for the separation of these phytochemical antioxidants. The majority of adsorption-HPLC techniques used for the analysis of carotenoids employ a silica stationary phase [59].

Separation of saponified carotenoids was carried out on a silica column (250 × 4.6 mm ID, 5 μm) using gradient elution from 95% light petroleum to 95% acetone [60]. With RP-HPLC, C_8 and C_{18} columns have been proved to be well suited for routine separations of carotenoids [61–63]. For more complex samples, particularly those high in esters, a C_{30} column seems to have better separation and selectivity than the conventional C_8 and C_{18} materials. A reversed-phase C_{30} column is particularly a good choice for the separation of geometric isomers of carotenoids [64]. Coupled C_{18} and C_{30} columns were also used to separation of carotenoids in various complex natural samples [65]. Figure 10.6 shows the separation of some carotenoids in various conditions by use of one column and two serial connected columns.

Better separation of the hydrocarbon carotenoids was possible with the polymeric C_{30} column. With monomeric C_{18} columns, lycopene usually elutes before α- and β-carotene, whereas with polymeric C_{18} and C_{30} columns, lycopene is strongly retained and elutes after these carotenoids [66]. Most recently, using C_{30} for liquid chromatography–mass spectrometry (LC–MS), Breithaupt were able to identify eight regioisomeric monoesters in addition to known lutein mono- and diesters [67]. Geometric isomers of free carotenoids have been separated using mainly C_{30} columns; however, the method using a reversed-phase C_{18} column in combination with DAD and mass spectrometric detection was recently developed, and for the first time, separated several *cis* isomers of lutein diesters [68].

10.5.4 Alkaloids

Gradient HPLC methods are very useful in alkaloid determination. A complete review of chromatographic and electrophoretic methods for analysis of phenetylamine alkaloids from *Citrus aurantium* is given by Pellati and Benvenuti [69]. Synephrine, octopamine, and tyramine were separated in

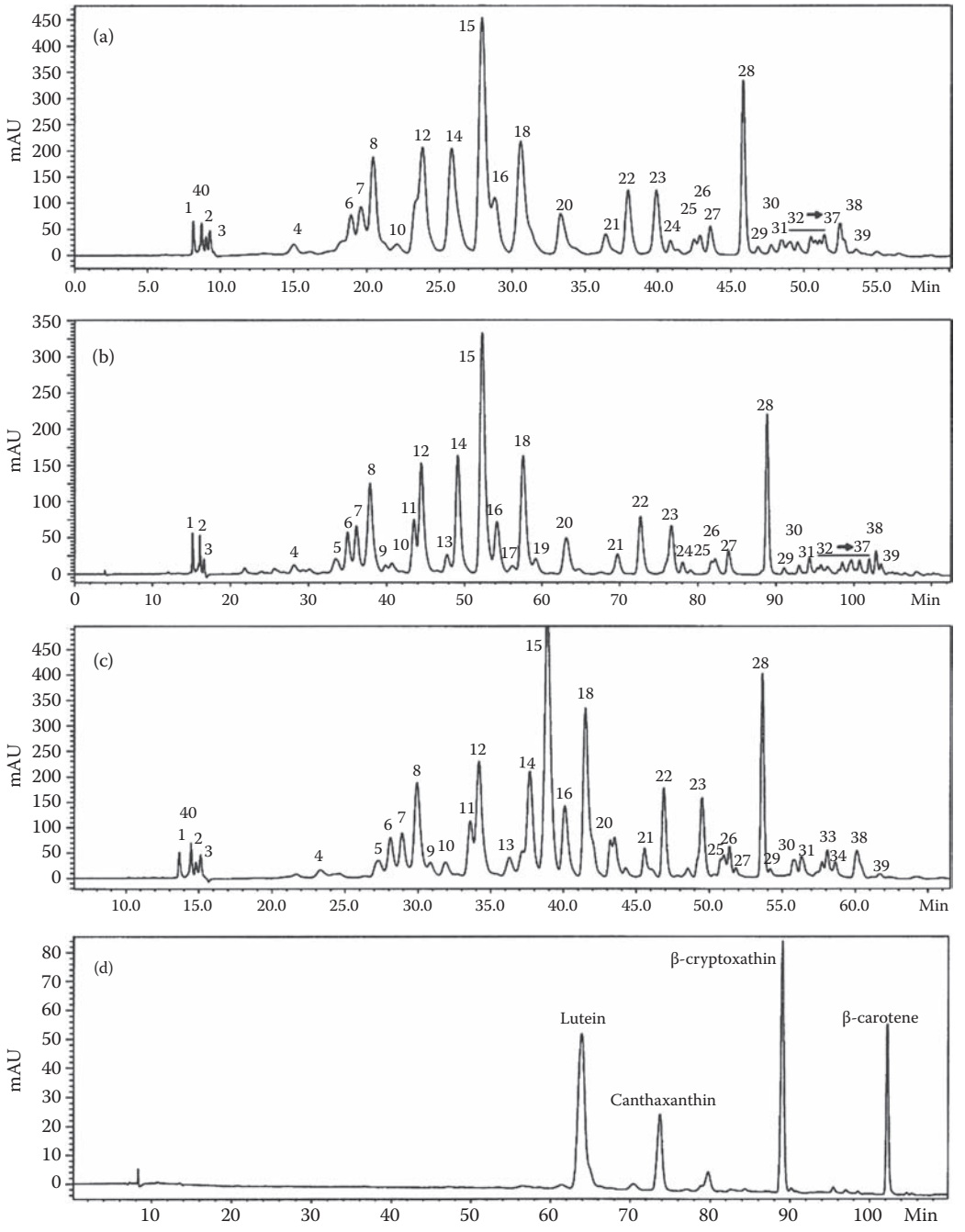

FIGURE 10.6 Chromatograms obtained from the separation of saponified red orange essential oil in (A) one C_{30} column, (B) two serial connected C_{30} columns doubling the gradient step times, and (C) two serial connected C_{30} columns keeping the one-column gradient. (D) Carotenoid standards separated using two serial connected C_{30} columns under the conditions employed in B. Wavelength showed 450 nm. Mobile phases: Solvent A, composed of methanol:water (90:10, v/v) and solvent B, composed of methanol: Methyl test-butyl ether (MTBE) (50:50, v/v), using the following gradient elution (flow rate: 0.8 mL/min): Starting from 0% B to reach 25% B at 5 minutes, then increasing to 50% B at 30 minutes, finally reaching 100% B at 50 minutes, maintaining these conditions for 10 minutes. (Reprinted from Herrero, M., Cacciola, F., Donato, P., Giuffrida, D., Dugo, G., Dugo, P., and Mondello, L., *J. Chromatogr. A*, 1188, 208–215, 2008. With permission.)

various gradient systems by use of C_{18} columns and buffers with acetonitrile and/or methanol as mobile phases.

An analysis of *Cinchona* alkaloids was presented by McCalley [70]. Various techniques, stationary phases, and mobile phases were discussed. Methods using HPLC, gas chromatography, thin-layer chromatography, and capillary electrophoresis (CE) were described. HPLC separations were shown using various types of columns (reversed-phase, cyano, amido) and gradients with buffers and acetonitrile as mobile phases. The current methods for chromatographic separation and determination of tropane alkaloids were summarized by Dräger [71]. Stockigt described HPLC and CE methods analysis of selected alkaloid groups [72].

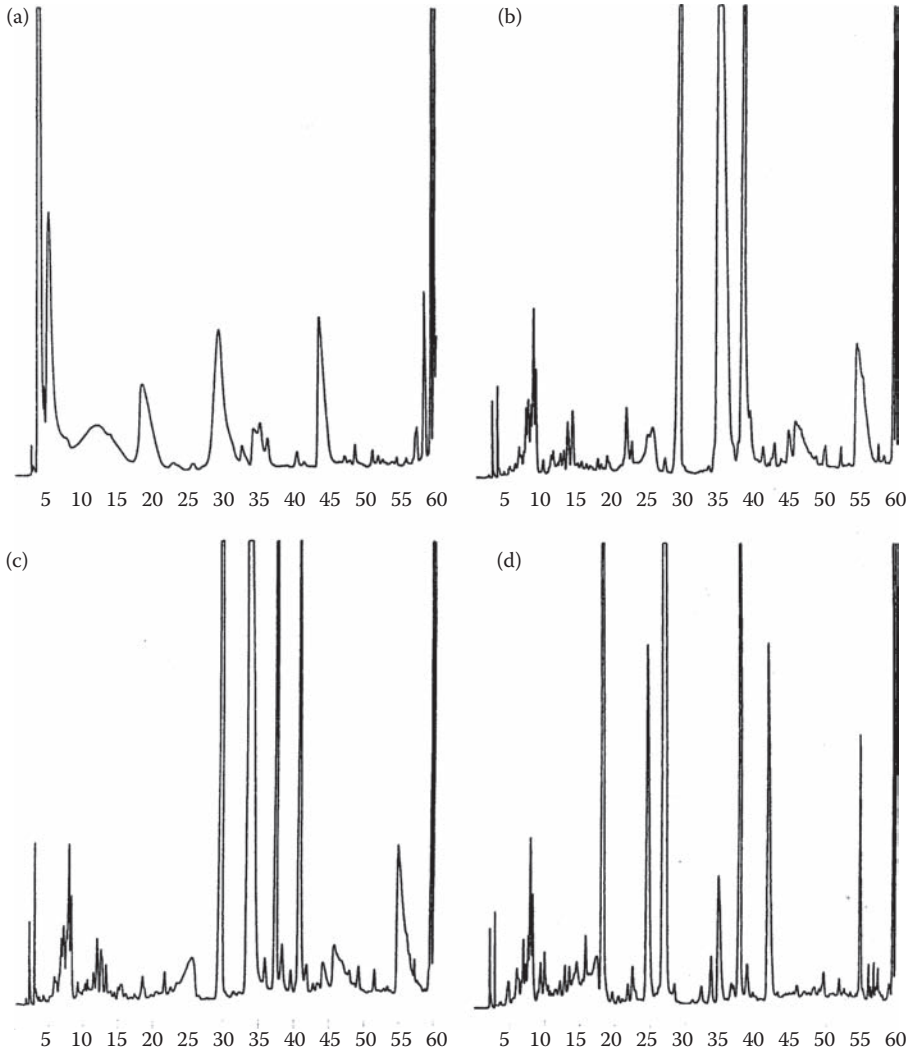

FIGURE 10.7 HPLC separation of the extracts of *R. serpentina x R. stricta* hybrid cell cultures: (a) acetonitrile:3.9 m*M* NaH_2PO_4 / 1.25 m*M* hexanesulfonic acid buffer (pH 2.5); (b) acetonitrile:3.9 m*M* NaH_2PO_4 / 1.25 m*M* hexanesulfonic acid buffer (pH 4.4); (c) acetonitrile:3.9 m*M* NaH_2PO_4 / 1.25 m*M* hexanesulfonic acid buffer (pH 5.5); (d) acetonitrile:3.9 m*M* NaH_2PO_4 / 2.5 m*M* hexanesulfonic acid buffer (pH 2.5); gradient 15:85 to 20:80 within 5 minutes, 25:75 within 20 minutes, 40:60 within 20 minutes, 80:20 within 15 minutes, flow rate: 1 mL/minute. (Reprinted from Stockigt, J., Sheludko, Y., Unger, M., Gerasimenko, I., Warzecha, H., and Stockigt, D., *J. Chromatogr. A*, 967, 85–113, 2002. With permission.)

Figure 10.7 presents the separation of some alkaloids from extracts of *R. serpentina x R. stricta* hybrid cell cultures.

10.5.5 Saponins and Steroids

Many methods of HPLC separation and determination of plant saponins were presented by Oleszek [73,74]. Dinan et al. [75] described chromatographic procedures for the isolation and analysis of various plant steroids: alkaloids, brassinosteroids, bufadienolides, cardenolides, cucurbitacins, ecdysteroids, saponins, withanolides, sapogenins, and glycoalkaloids. A total-ion-current (TIC) chromatogram (upper) of *Medicago truncatula* saponins and a selective ion chromatogram (SIC) for the mass of *m/z* 1251 are presented in Figure 10.8. Analysis methods for ginsenosides and other groups of active compounds in *Ginkgo biloba* leaves and extracts were also presented [76,77].

10.5.6 Other Compounds

Valepotriates (valeriana-epoxy-triesters) are natural products that, from a chemical point of view, belong to the iridoids. The determination and analysis of valepotriates was reviewed by Bos et al. [78].

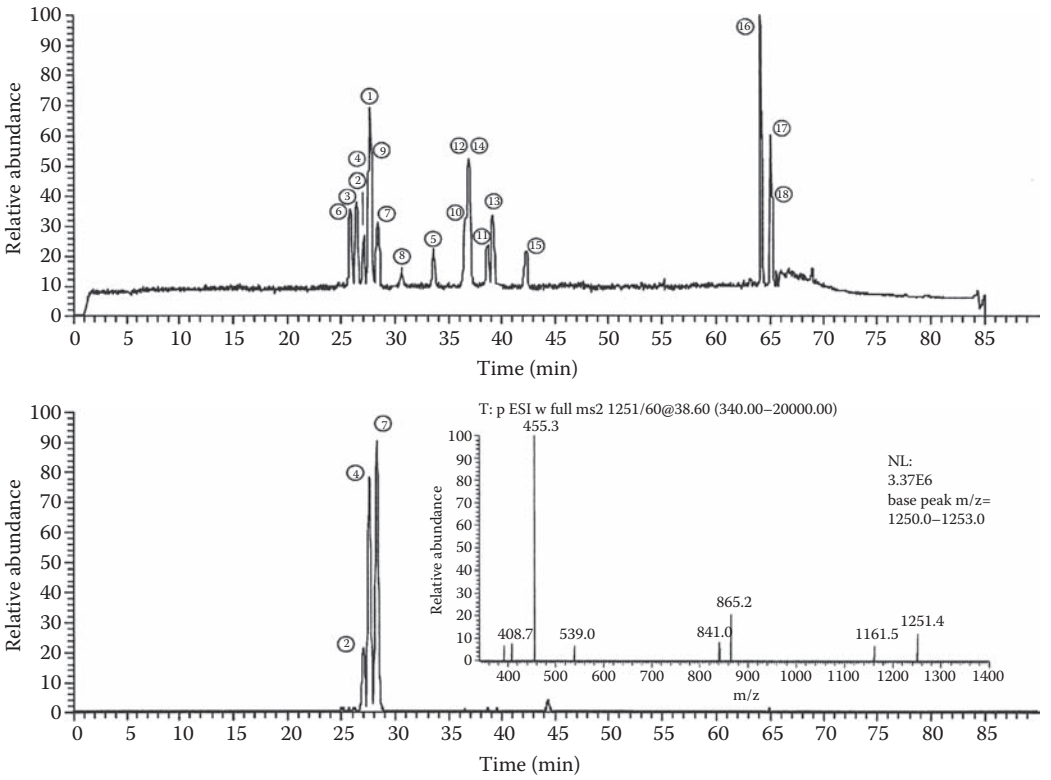

FIGURE 10.8 Total-ion-current (TIC) chromatogram (upper) of *Medicago truncatula* saponins and selective ion chromatogram (SIC) for the mass of *m/z* 1251. Compounds: (2) 3-*O*-β-Glc-(1→3)-β-Glc,28-*O*-β-Api-(1→3)-α-Rha-(1→2)-α-Ara-zanhic acid; (4) 3-*O*-β-Glc-(1→3)-β-Glc,28-*O*-α-Ara-(1→3)-α-Rha-(1→2)-β-Ara-zanhic acid; (7) 3-*O*-β-Glc-(1→3)-β-Glc,28-*O*-β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara-zanhic acid. LC conditions: Finnigan Surveyor pump with gradient controller, autosampler, and PDA detector; column 250 × 4 mm ID, 5 µm, Eurospher 100 C_{18}; mobile phase: solvent B 0.05% acetic acid in water, solvent A 0.05% acetic acid in acetonitrile; flow rate 0.5 mL/min; run time 90 min; gradient program: from 18 to 36% A in 55 minutes, from 36 to 100% A in 20 minutes; injection volume 25 µL. (Reprinted from Oleszek, W. and Biały, Z., *J. Chromatogr. A*, 1112, 78–91, 2006. With permission.)

Polyprenols (long-chain alcohols) are substances that can be found in plants, animals, and other eukaryotic organisms. They also occur in some bacteria cells. The protective role of polyisoprenoids against reactive oxygen and nitrogen species has induced interest in these lipids on the part of the pharmaceutical industry. A review of the analytical techniques for polyprenols from animal and plant sources was presented by Rezanka and Votruba [79].

Various methods of analyzing sesquiterpenes and sesquiterpene lactones were described by Russo et al. [80] and reviewed by Merfort [81]. Separation techniques and procedures for the pharmacologically active components of various plants (e.g., rhubarb, onion and garlic, piper, gardenia) and other groups of active compounds (e.g., xanthones, quinoids) have also been reviewed, as well as separation techniques applied in phytochemistry [82–93].

10.6 COMPUTER-ASSISTED METHOD DEVELOPMENT

Complex, multicomponent mixtures, such as extracts from medicinal plants, are very difficult to separate in one isocratic chromatographic run. For this reason gradient elution should be optimized for these separations. In the past a trial-and-error method was used to optimize gradients in all HPLC analyses. Nowadays, computers are powerful tools for predicting separations as a function of experimental conditions.

Computer-assisted optimization of parameters has not been universally accepted, primarily due to a lack of ease of use. All compounds must be tracked across all experiments, and all retention times must be entered into the system for each component. This is sometimes difficult because significant variations in the retention and elution order could be observed for certain analytes. Advantages of this technique are the efficiency of method development, structured development profiles, and effective reporting of what was performed during the different method-development interactions.

There are several methods and computer programs used in gradient optimization, such as DryLab, ChromSword, OSIRIS, PREOPT-W, HEUREKA, AMDS, AutoChrom, ACD/LC Simulator, ACD/ChromGenius, EluEx, and ChromaSmart.

10.6.1 DryLab Software

DryLab chromatography-modeling software takes the trial-and-error out of method development. This software simplifies and speeds the process of developing good chromatographic separations or methods by allowing one to model changes in separation conditions using a personal computer. The time-consuming and repetitive laboratory runs that are typically required to achieve a satisfactory separation or a complete method are replaced with instantly generated chromatograms that correspond to the selected separation conditions. A wide range of conditions can be modeled in minutes—more than would ever be practical to try in the laboratory. DryLab bases its modeling on two or more "calibration" runs that one carries out on a chromatograph. The detailed separation results generated by DryLab are based on thoroughly tested algorithms. An extensive list of literature references describes the theoretical background of DryLab and its applications.

DryLab has dozens of uses in the chromatography laboratory: finding a good set of separation conditions quickly, optimizing a method in minimal time, predicting method robustness, troubleshooting existing methods, shortening run times while maintaining adequate resolution, transferring methods to other instruments, validating methods, and finding the best separation conditions for any component in a complex mixture.

DryLab includes modeling options for reversed-phase isocratic percentage of B, gradient liquid chromatography, the optimum pH, pH, isocratic ternary conditions, ionic strength, additive/buffer concentration, liquid chromatography temperature, gas chromatography temperature program, normal-phase isocratic percentage of B, reversed-phase gradient and temperature, reversed-phase

isocratic percentage of B and temperature, reversed-phase gradient and pH, reversed-phase isocratic percentage of B and pH.

In addition, DryLab shows the effect of changes to column dimensions, particle size, flow rate, and dwell volume for the separation.

10.6.1.1 Theoretical Basis

The DryLab software is based on some semiempirical concepts:

1. In RP-HPLC systems, retention can be presented as

$$log\ k\ =\ log\ k_w – S\Phi, \tag{10.1}$$

   where k_w and S are empirical constants for a given solute and experimental conditions and Φ is the volume fraction of organic solvent B in the mobile phase [94].
2. Based on the equation

$$\log k = \frac{a+b}{T_k} \tag{10.2}$$

   with only two-experimental runs, it is possible to predict isocratic retention as a function of Φ and gradient retention as a function of gradient conditions, column size, and flow rate [95]; together, Equations 10.1 and 10.2 comprise what has come to be called the linear-solvent-strength model of HPLC [96].
3. For either isocratic or gradient elution it is possible to approximate bandwidth by means of the Knox equation, when the sample molecular weight, temperature, mobile-phase composition, and "column conditions" are known.

The accuracy of DryLab, based on the preceding relationships, has been analyzed in many papers [10,13,14,97–101]. In most cases, the accuracy of resolution predictions is of the order of ±10%, which is usually more then adequate. Expressions such as Equations 10.1 and 10.2, which are suitable for predicting retention as a function of certain conditions, require only two-experimental runs for DryLab implementation. Other variables, such as pH or buffer concentration, require three or more runs, in which cases a cubic spline fit to the data can be used for predictive purposes [102].

10.6.1.2 Practical Applications of DryLab

The effective use of DryLab requires some reasonable first choices of experimental conditions (column packing, mobile-phase solvents, buffer, pH, etc.) [96]. DryLab software was used in many cases of phytochemical analyses. Korner and Kohn used monolithic reversed-phase column for the optimization of the separation of Colchicum dry extract [103], and gradient separations of flavonoids aided by DryLab software have also been described [104,105]. Computer-assisted optimization of the development of HPLC for kava pyrones was performed by Schmidt and Molnar using DryLab [106], and antraquinones were analyzed by Liu [107] and Metzger and Reif [108]. Some coumarins from *Archangelica officinalis* were separated by Hawryl using gradient HPLC and TLC systems in which HPLC experiments were optimized by DryLab [109]. Hajnos developed a method for the separation of some taxoids from yew extracts by use of DryLab optimization and gradient HPLC [110]. Molnar [111] comprehensively reviewed the development of the DryLab software.

Figures 10.9 and 10.10 show four original chromatograms with different t_G/T values and a plot of critical resolution against temperature and gradient time using 2-propanol as the organic modifier for the separation of kava pyrones in *Piper methysticum* preparations.

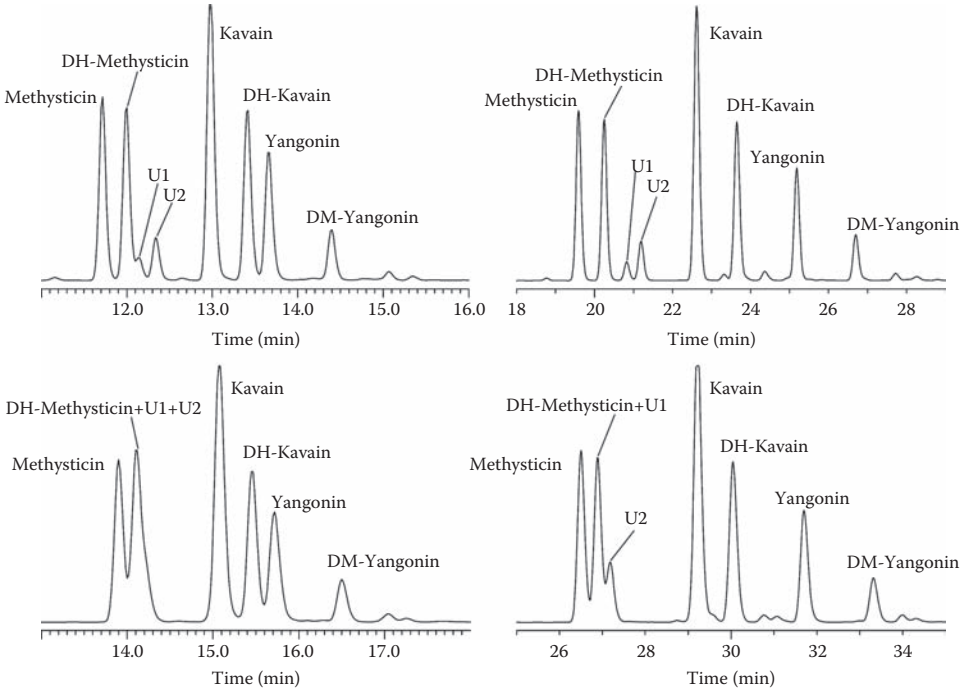

FIGURE 10.9 Four original chromatograms with different t_G/T values using 2-propanol as the organic modifier. Lower left: 30 min/30°C; lower right: 90 min/30°C; upper left: 30 min/60°C; upper right: 90 min/60°C. (Reprinted from Schmidt, A. H. and Monar, I., *J. Chromatogr. A*, 948, 51–63, 2002. With permission.)

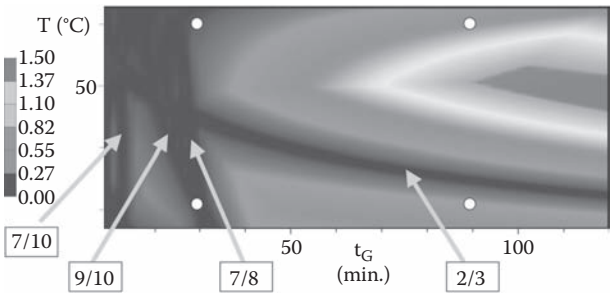

FIGURE 10.10 Plot of critical resolution against temperature and gradient time. Eluant B was 2-propanol. There is a fairly rugged region at T 50–60°C and t_G 100–200 minutes. The analysis time is about 25 min (T 50°C; t_G 70 minutes). Using 2-propanol, an acceptable baseline separation was obtained. (Reprinted from Schmidt, A. H. and Monar, I., *J. Chromatogr. A*, 948, 51–63, 2002. With permission.)

10.6.2 ChromSword Software

ChromSword is a software program for computer-assisted HPLC method development and optimization. Based on physicochemical retention models or empirical HPLC data and using various optimization and simulation procedures, chromatographic separations are optimized with respect to peak resolution and analysis time. ChromSword helps to save considerable time and effort in the method-development process.

This process has more advantages:

- Virtual chromatography: For the optimization of reversed-phase methods, optimum conditions are immediately predicted after entering the compound's structural formula

and the column properties. This reduces the number of necessary experiments to a minimum.
- Empirical approach: Alternatively, all optimizations can be performed using the data from a minimum of two-initial experiments at different conditions.
- Superfast gradient optimization: Linear and multistep gradients are optimized quickly using a superfast Monte Carlo optimization process.
- 2D optimizations: Using two-dimensional resolution maps, two-chromatographic parameters can be optimized simultaneously, allowing analysis of resolution to determine the limiting factor for peak resolution.

10.6.2.1 Theoretical Basis

The main equation derived for calculating retention in reversed-phase chromatography is given as

$$\ln K = a(V)^{2/3} + b(\Delta G) + c, \tag{10.3}$$

where k is the retention factor, V is the partial molar volume of the modifier in water, ΔG is the energy of the interaction with water, and a, b, and c are the parameters of the sorbent/eluent system [112–115].

After compounds' structures have been entered, the software calculates values for V and ΔG automatically. The a, b, and c parameters are obtained by calibrating the solvent/eluent system with a suitable set of reference standards. The software contains a database of these parameters for a variety of commonly used columns. According to the entered formula, the theoretical retention model is calculated for each compound to be separated. This model is used to predict the optimum starting conditions for the experimental method-optimization process. Experimental results show that in most cases the predicted optimum organic-modifier concentration differs from the real optimum by a maximum of ±5–6%. If the structures of the analyzed compounds are unknown, RP-HPLC methods are optimized using the empirical approach.

The software has been equipped with a function to be able not only to perform experiments with one column/organic modifier combination but also to automatically optimize a method with different column/organic modifier combinations. If two reversed-phase columns with different properties (e.g., RP-8 and RP-18) and two different organic modifiers (e.g., methanol and acetonitrile) are applied, the system performs an automatic method-optimization procedure for each column/eluent combination [116].

10.6.2.2 Practical Applications of ChromSword

ChromSword has been applied to optimize many RP-HPLC systems that have been applied in pharmaceutical analysis [117,118,119], environmental analysis [120], and biomedical analysis [121]. A comparison of DryLab and ChromSword in isocratic separations of neutral compounds has been described by Bączek [122].

The final gradient chromatogram for lamotrigine using automated screening followed by ChromSword-Auto automated HPLC method development is presented in Figure 10.11.

10.6.3 Other Software and Computer-Assisted Methods

10.6.3.1 OSIRIS

Another software for the optimization of HPLC conditions is OSIRIS. The model (as for DryLab and ChromSword) is derived from a few preliminary runs. Both their number and type depend on the chosen mobile-phase system, on the composition zone explored, and eventually on the temperature zone or the pH zone (in case of ionizable compounds). The results (retention times of each compound) and the chromatographic data from these preliminary runs have to be entered in the OSIRIS project file.

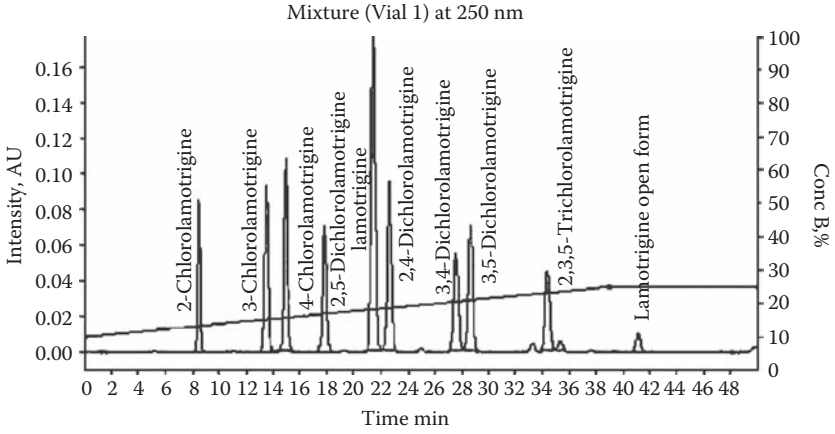

FIGURE 10.11 Resultant gradient chromatogram for lamotrigine using automated screening followed by ChromSword-Auto automated HPLC method development. *Experimental conditions*: column: Inertsil ODS 3 (100 × 4.6 mm; 5.0 µm); flow rate: 1.5 mL/min; UV detection: 250 nm; column temperature: 40°C; gradient mobile-phase composition: 0 min, 10% B; 39 min, 25% B; 50 minutes, 75% B. Solvent A: 1.0% (v/v) triethylamine, pH 2.0; solvent B: acetonitrile. (Reprinted from Hewitt E.F., Lukulay, P., and Galushko, S., *J. Chromatogr. A*, 1107, 79–87, 2006. With permission.)

This phase is essential in the optimization process because the optimum validity primarily depends on the model accuracy. The retention model will become more accurate as the parameter space is reduced. For this reason, in order to optimize a ternary or quaternary mobile phase, OSIRIS uses a specific technique that searches for the optimum in the most favorable composition zones. These zones are determined by OSIRIS using the CRIT parameter. The CRIT parameter is a unique tool that allows the most favorable composition zones for ternary and quaternary mobile phases to be selected. This limitation of the explored zones reduces the number of necessary preliminary experiments and improves the accuracy of the model.

Five optimization processes are available in the software: pH optimization (parameter, 3 experiments), temperature optimization (1 parameter, at least 2 experiments), mobile-phase optimization (from 1 to 3 parameters, between 2 and 12 experiments), mobile-phase and temperature optimization (2 or 3 parameters, between 4 and 10 experiments), and mobile-phase and pH optimization (2 parameters, 9 experiments).

The software has been used in the optimization of the separation of some ionizable compounds [123] and polycyclic aromatic hydrocarbons (PAHs) [124].

10.6.3.2 PREOPT-W

PREOPT-W is another Windows software package specifically designed for the optimization of binary gradients in HPLC. Cela et al. [125–128] have demonstrated that any gradient profile (linear, curve, or stepwise composite gradients) can be accurately simulated by a series of isocratic steps of increasingly modified proportion, provided the increase in modifier percentage is produced with sufficient speed. This software produced models specifically designed for each separation problem. This means that biased models will be built up that will remain useful while the chromatographic system and the problem mixture remain unmodified. Retention of compounds is optimized departing from isocratic retention data at various modifier percentages. Cubic splines fit is used to interpolate retention values inside this modifier range. From this retention model, it is possible to calculate the retention time of each compound under any gradient conditions, thus simulating gradient chromatograms. Polyphenols were successfully separated through use of PREOPT-W gradient simulation [129,130].

10.6.3.3 Other Methods of Computer-Assisted Method Development

There are numerous software programs and methods to predict retention and resolution in HPLC. The CHROM software (an algorithm for modeling retention times in gradient RP-HPLC) has been described by Vivo-Truyols [131–133]. The authors optimized the resolution of 16 β-blockers using both isocratic and gradient training sets written in MATLAB® software.

A new approach, based on the Snyder theory, consists of the computer-assisted selection of mobile phases for the separation of nonionic compounds by normal-phase chromatography; this was described by Palamarev et al. [134,135]. The authors developed software called LSChrom. This application enables one to characterize mobile phases with the three parameters from the Snyder theory: mobile-phase strength, localization ability, and polarity.

A software program called Chromatogram Generator was developed by Pirogov et al. [136]. This software allows the approximation of chromatographic peaks with several mathematical functions and their superpositions, the construction of series of chromatograms with various parameters, and the construction of a graphical image of a chromatogram with minimum size and the ability to be added to chromatographic database.

PREGA is a package of programs developed to allow the off-line optimization of HPLC separations [137]. Departing from a few experiments, PREGA develops a retention model for the compounds in the mixture to be separated. That model will be used to predict chromatograms, thus enabling the optimization of isocratic and gradient separations. Practically any type of gradient can be explored (linear, curved, multilinear, and stepwise gradients are available). Moreover, additional variables such as the temperature and mobile-phase flow rate can be manipulated and optimized by the package. PREGA is highly interactive although friendly, allowing optimizations using the more conventional conditional random fields (CRF) functions but also, for the first time in chromatography, Pareto optimization. Currently, modules for binary mobile phases are available. In the last version, algorithms for transferring gradient program elutions from instrument to instrument or column to column were incorporated.

Jin et al. established a new method for predicting retention times and peak-shape parameters of compounds with unknown structure in complex samples (*Rhizoma corydalis*, *Salvia miltiorrhizae*) under linear gradient mobile-phase conditions [138–141]. On the basis of an equation that relates chromatographic peak shape ($W_{h/2}$) to retention values (t_R), authors developed a complex sample-analysis software system (CSASS) to calculate and predict gradient conditions for the separation of 24 compounds.

Multiple linear regression (MLR), an artificial neural network (ANN), and a solvation parameter model were used by Fatemi et al. and by Chu and Poole to predict gradient retention times [142,143].

Some novel methods and training software for the prediction of chromatographic conditions in gradient HPLC and capillary electrophoresis were also described by Reijenga and colleagues [144,145].

There are many publications in which authors compare many software programs for prediction of retention and other parameters in gradient HPLC [146–151]. Some applications of computer-assisted methods in phytochemical analysis have also been described [138–141,152,153].

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11 LC-MS as a Method of Identification and Quantification of Plant Metabolites

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CONTENTS

11.1 INTRODUCTION

Plants and extracts there of have been used in traditional medicine for the treatment of various diseases for centuries all over the world, besides their use in food and cosmetics. In fact, already in the ancient world, the use of plant extracts as a kind of medicinal therapy was described. Nowadays they are still indispensable in various countries with no or only limited access to Western medicine due to the lack of commercially available drugs. The interest in characterizing and isolating bioactive constituents of plant extracts for the development of new medicines in the fight against various diseases like malaria and in the search for new antibiotics is increasing again and displays a huge potential with a large business market. For this reason, the analytic techniques used for the characterization of plant constituents—for example, in the quality control of herbal medicinal products as well as in the segment of high-priced food products—have moved into the spotlight. Due to the complexity of primary and secondary metabolites present in plants, new ground has to be broken in the development of sophisticated analytical approaches and tools, which presents a major challenge with respect to the analytical point of view. In particular, the composition of complex plant matrices asks for adequate methodologies like mass spectrometry (MS).

The mass spectrometer, invented over a hundred years ago, is one of the most important tools to determine molecular mass, structure, isotope composition, and relative or absolute abundance. With a special focus on plant-ingredient analysis via MS, this chapter is divided into two parts: a technical part summarizing main developments and state-of-the-art instrumentations and an application part focusing on different interesting research fields.

11.2 PRINCIPLES AND INSTRUMENTATION

Today a variety of mass spectrometers are available. Due to the complexity of metabolites as regards variability in their chemical and physical properties as well as their concentration, no analytical platform can answer all questions. Nevertheless, MS is a powerful tool that is gaining more and more importance. The functionality of a mass spectrometer can be seen as a four-step process [1], starting with sample inlet, transfer into gas phase by the ion source, the mass analyzer and finally the detector. This leads to two different moieties that are important for the user. Sample inlet and ion source define the requirements for sample concentration, matrix, and pretreatment. Mass analyzer and detection systems are defined by the manufacturer of the mass spectrometer and are normally not exchangeable.

Generally, a classification into off-line MS—such as direct-infusion mass spectrometry (DIMS) and matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS)—and on-line hyphenation of chromatography to MS—such as high performance liquid chromatography–mass spectrometry (HPLC-MS)—is possible. In HPLC-MS the so-called ion source accomplishes the transfer of the analytes from the solution at atmospheric pressure to the gas phase at vacuum pressure with simultaneous ionization. Examples of ion sources or so-called interphases are shown in Figure 11.1.

Electrospray ionization (ESI) belongs to the soft ionization techniques. Flow rates from μL min^{-1} to nL min^{-1} (with a nano source) are possible. The solution/solvent is sprayed out of a capillary into an electric field (1–5 kV). The resulting Taylor cone produces small droplets, which get smaller by evaporation of the solvent. The desolvatation and formation of ions takes place in the heated capillary. Mainly, two theories for the generation of single ions are discussed. The desolvatation of the ions can be supported by the application of sheath gas, dry gas, and curtain gas and also by the heated capillary. However, analyte molecules should end up completely solvent free and charged [2,3].

In *atmospheric pressure chemical ionization* (APCI), the solution or solvent is passed through a heated glass tube (up to 500°C), and, supported by high flow rates of nitrogen, an explosive vaporization is induced, generating a gaseous collection of solvent and analyte molecules. High voltage applied to a needle (corona discharge) enables the ionization of the solvent molecules, and consequently charge or ion transfer to the analyte molecules. APCI produces ions in the gas phase and belongs to the soft ionization methods, although it is more prone to fragmentation of analytes, caused by the high temperature, than ESI is. The direct connection of standard-bore HPLC is possible without prior split. APCI is suited for polar and semipolar metabolites that are thermically stable and lack easily ionizable functional groups such as alcohols or ketones. Examples of compounds where APCI is used are triglycerides and carotenoids.

For *atmospheric pressure photoionization* (APPI) the solvent is vaporized by the application of nebulizer gas and a heater. Subsequently, ionization is induced by photons from a discharge ultraviolet (UV) lamp. This technique can be applied for the ionization of nonpolar but also polar compounds and is mainly used if analytes show poor charge affinity, as do steroids and vitamins.

The process of ionization and desolvatization delivers a number of different *m/z* values, due to different charge states and molecules. Therefore, mass separation and consecutive detection are necessary, which can be achieved by a number of different principles and their combinations.

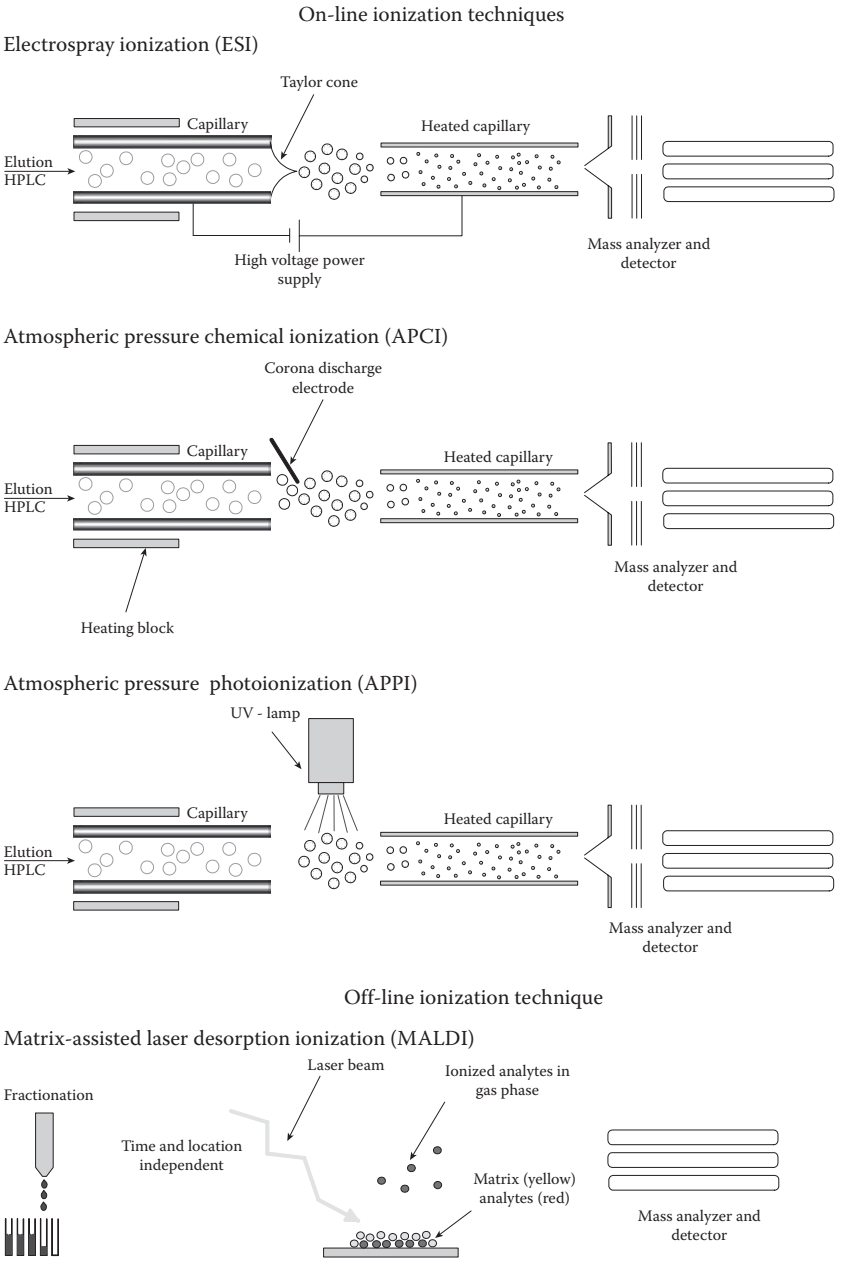

FIGURE 11.1 On-line and off-line techniques for hyphenation of HPLC to mass spectrometry.

Generally, three types of mass analyzers are described as being employed for the separation of single *m*/*z* values: quadrupole, ion trap, and time of flight (TOF); see Figure 11.2.

The transfer of ions to the gas phase in the off-line mode is explained shortly on the basis of the soft ionization technique MALDI. After fractionation of samples via, for example, HPLC, aliquots are embedded into a crystalline matrix (Figure 11.1). This matrix absorbs irradiated laser energy and therefore protects analytes from direct laser beams. Furthermore, it facilitates vaporization and ionization of analytes.

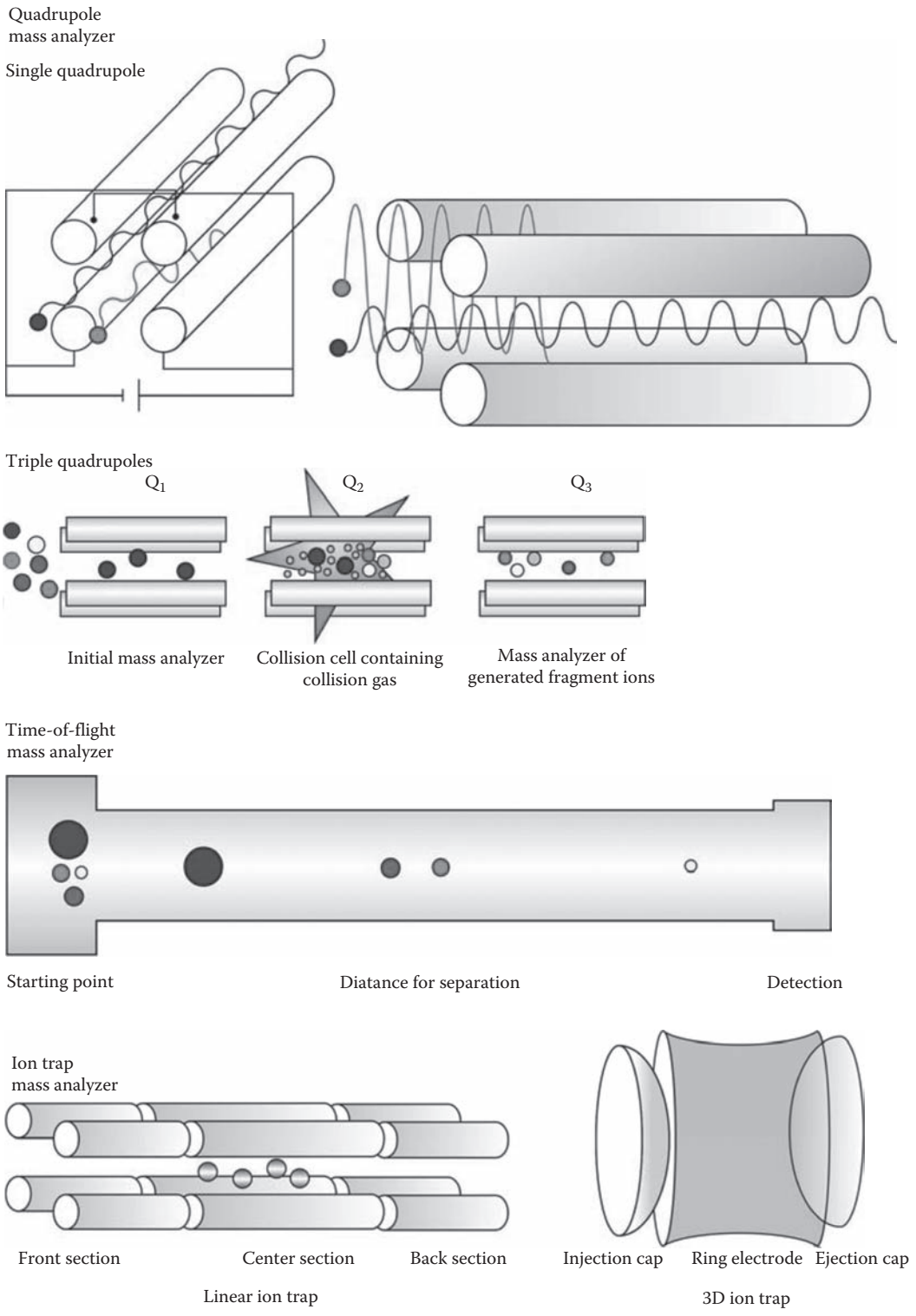

FIGURE 11.2 Main types of mass analyzers used in phytochemical approaches.

Karas, Bachmann, and Hillenkamp coined the term MALDI in 1985 [4] and successfully demonstrated the use of small organic molecules as a matrix to circumvent certain existing limitations like molecular mass range and sensitivity. In a further development Tanaka et al. showed the application of MALDI by analyzing macromolecules [5]. The fundamental processes like desorption and ionization are still poorly understood. Generally, the desorption process can be defined as excitation of analyte molecules, the subsequent phase change, and the dynamics of the material plume expansion. Laser parameters (wavelength, pulse duration, and laser fluence) and the dynamic parameters of the expanding MALDI particle plume play a very important role in the desorption process. Further, the thermodynamic properties of matrices and matrix–analyte interactions in the sample are also crucial and play a decisive role during analysis. In fact, sufficient rapid phase transitions from the condensed to the gas phase and gas-phase interactions, indicated by the dynamic properties of the expanding plume, are essential [6]. In addition, the initial temperature and pulse duration also play a big role, as shown by Zhigilei and Garrison in a molecular dynamic simulation model for the ablation/desorption process [7,8]. Theoretical models for the ionization as well as molecular modeling suggest that this process generates clusters and material particles besides gaseous components.

Zenobi and Knochenmuss have divided the MALDI ionization process into two categories: primary and secondary ionization mechanisms [9,10]. The motivation of this classification has been based on the time scale of the laser pulse (3–5 ns) and the time required for expansion to collision-free densities (many μs) [11]. It is assumed that primary ions are generated during or shortly after the laser pulse, or within the excited-state lifetime of the matrix. These ions are often matrix-derived species [9]. Thus the photoionization of matrix molecules is assumed as the initial step in the ionization process. Primary ionization events are somewhat controversial and are mainly assumed to have gone through the photoionization and cluster-formation mechanism [10]. Primary ionization events are followed by secondary ionization reactions in the expanding plume. During secondary reactions a charge transfer to the analyte molecules takes place. This transfer continues as long as there are fruitful collisions between ions and neutrals, which lead to the reaction [11].

After ionization and transfer of the sample into the gas phase, the analytes are separated or resolved according to their mass-to-charge ratio through mass analyzers. In fact, all mass spectrometers are based on the dynamics of charged particles in electric and magnetic fields in a vacuum following the Lorentz force law and Newton's second law of motion.

The following presents an overview of the mass analyzers most often used in phytochemistry (Figure 11.2).

Quadrupole-Based Instruments. *The quadrupole mass analyzer was developed by Paul and Steinwedel [12]. It can be described as a mass filter. The combination of AC and DC voltages applied to four parallel rodlike electrodes allows ions of certain m/z values to pass through on stable oscillating pathways, whereas ions of different m/z values are removed by collision with rods. With appropriate voltage settings a wide range of m/z values can also be guided by a quadrupole; therefore, they are used in many mass spectrometers to transport ions from one analyzing step to another. In addition, hexapoles and octapoles are used, basing on the same principle, with slightly different properties regarding ion filtering and transportation*. Generally, single-quadrupole instruments and triple-stage-quadrupole instruments are available.

Single-quadrupole instruments consist of the ion source, a single quadrupole, and a detector. The AC and DC voltages on the electrodes of the quadrupole are defined, enabling only specific *m/z* values to reach the detector. Single-quadrupole instruments provide only MS spectra with low resolution and are the cheapest instruments on the market. Generally, this type of instrument is used in combination with several chromatographic separation systems like gas chromatography or liquid chromatography (LC). In particular, single-quadrupole mass spectrometers are a fundamental part of semipreparative separation systems.

Triple-quadrupole (QQQ) or triple-stage-quadrupole (TSQ) instruments can be used in the same manner as a single quadrupole, using the first or the third quadrupole as the mass filter and the other only for ion transfer through the instrument to the detector. For fragmentation

experiments, called MS/MS experiments, the first quadrupole is used as a scanning device, allowing the selection of precursor ions. Selected ions are fragmented in the second quadrupole by a collision gas (mostly Ar or N_2), which is used as the collision cell. The third quadrupole is used to scan the product ions and give information about the generated fragments. Another possibility for a QQQ is the precursor ion scan. In this working mode the first quadrupole scans for a specific *m/z* range, fragmentation is performed in the second quadrupole, and the third quadrupole scans for specific product ions. Furthermore, a scan for constant neutral loss can be performed. In this mode, the first and third quadrupole scan in parallel but with the difference of a specific neutral loss mass, enabled by fragmentation in the second quadrupole. Only ions that show a specific mass loss by fragmentation are detected. In selected reaction monitoring (SRM) experiments, two mass analyzers are used as static mass filters to monitor a particular fragment ion of a selected precursor ion. In multiple reaction monitoring multiple SRM transitions can be measured within the same experiment on the chromatographic time scale by rapidly toggling between the different precursor/fragment pairs.

Ion Trap Instruments. The ion trap is a modified quadrupole with the capability to trap (store) ions on stable trajectories. Depending on the geometry of the electrodes and the resulting movement of the trapped ions, two- and three-dimensional ion traps distinguished. A three-dimensional ion trap consists of a ring electrode, which is closed by end cap electrodes at both sides. A detailed description of its working mode was given by March [13]. By controlling the voltages applied to the caps, containing cylindrical bores, the trapping of defined *m/z* values is possible.

The two-dimensional or linear ion trap consists of a quadrupole that is separated into a center and two end sections, to which different voltages can be applied. The ions are trapped in a two-dimensional field generated by the multipoles, permitting increased scan speed, storage capacity and thus the sensitivity compared to a three-dimensional ion trap. One or more quadrupoles are located after the ion source to transfer the ions into the trap, which allows storage and separation of ions, isolation of specific *m/z* values, collision-induced dissociation (MS/MS or MS^2), and also the re-fragmentation of product ions (multistage MS, or MS^n). Two-dimensional traps can be subdivided into two groups depending on the direction of the ions when scanned out. One possibility is to scan ions out radially to the central axis of the trap. This allows the use of two detectors at both sides of the trap and provides increased sensitivity (e.g., LTQ from Thermo Fisher Scientific, Waltham, Mass.). Alternatively ions can be scanned out axially. This allows the construction of mass spectrometers that combine the advantages of a QQQ with a linear ion trap [14], like the MDS Sciex/Applied Biosystems 4000 Qtrap.

The advantages of ion trap are their high sensitivity and the possibility to perform MS^n experiments. Admittedly it is not possible to perform precursor ion and neutral loss scanning with the same speed as with QQQs, and the generated mass spectra are low-resolution and of low-mass-accuracy.

Time-of-Flight (TOF) Instruments. TOF mass analyzers separate mixtures of ions based on the time they need to pass a field-free flight tube after being accelerated by voltage. To increase the precision of the separation, not only linear but also V- and W-shaped flight tubes are used.

Quadrupole–Time-of-Flight (Q-TOF) Instrument. Q-TOF instruments can be seen as a further development or the combination of a QQQ with a TOF instrument, providing high resolution with MS/MS possibility. The quadrupole is used for selection of precursor ions, which are fragmented in the collision cell; product ions are then analyzed with high resolution with the TOF mass analyzer. The instrument consists of an ion source, a quadrupole, a hexapole collision cell, and the TOF analyzer with ion pusher, reflectron, and detector [15]. Actual Q-TOF instruments provide scan speeds of 20 Hz with a resolution of 20,000. The latest improved version is the Maxis made by Bruker (Bremen, Germany), which provides a resolution of up to 60,000.

Besides the standard detectors (not described here) two very important technologies, the Fourier transformation ion cyclotron resonance (FT-ICR) and the latest development, the Orbitrap, serve as both a mass analyzer and a detector.

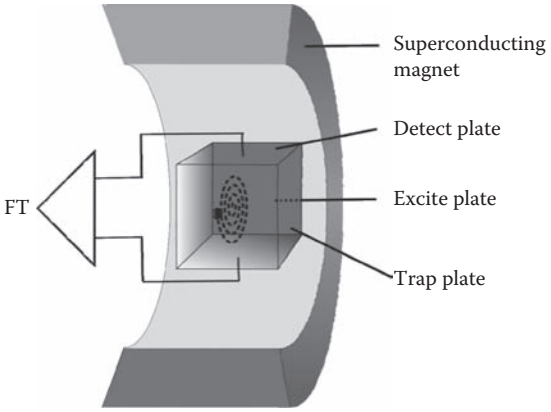

FIGURE 11.3 Fourier-transform ion cyclotron resonance (FT-ICR) mass analyzer.

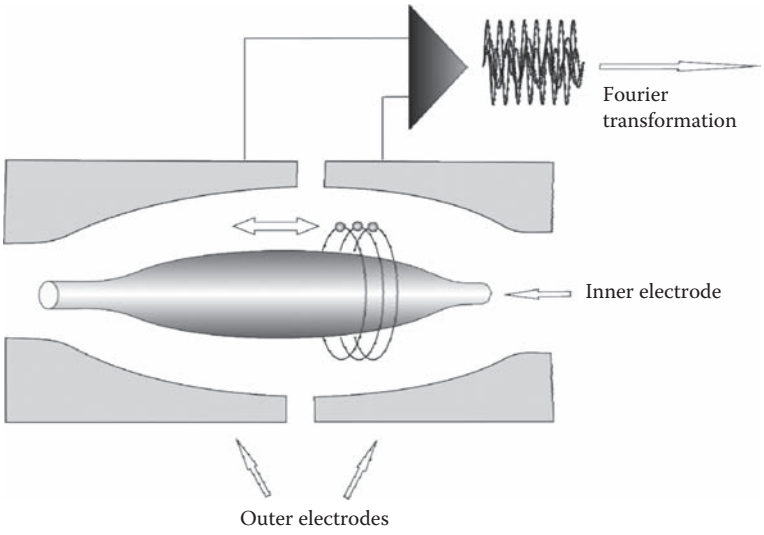

FIGURE 11.4 Orbitrap mass analyzer.

FT-ICR. The ions are injected into a Penning trap and forced on stable trajectories with specific cyclotron frequencies by a very strong fixed magnetic field up to 15 tesla. Through the application of an oscillating electric field, the ions are excited to larger cyclotron radii and also move in phase (in a packet), producing an image current on a pair of plates at the sides of the Penning trap. From this signal, m/z values are calculated by the performance of Fourier transformation.

Due to the high-mass accuracy and resolution together with low-detection limits, FT-ICR is a perfect tool for the investigation of metabolites [16]. Metabolites can be fully mass resolved, and a high percentage can be identified. The power of the instrument is constantly being improved further [17]. The application of ESI and APCI in positive and negative mode together with FT-ICR enabled Aharoni to detect 5844 unique ^{12}C single charged masses from ripening strawberry fruit, with more than half of them identified [18]. FT-ICR mass spectrometrs are the instruments with the highest resolution but also the highest price.

Orbitrap. The working principle of the Orbitrap is similar to that of the FT-ICR. The mass spectrometer is an electrostatic ion trap and consists of an inner spindle-like electrode, which is surrounded by two outer barrel-like electrodes with an electric field applied between them. Ions orbit

the central electrode in axial and radial directions, through a balance of electrostatic and centrifugal forces, and the orbital frequency is detected as an image current by the outer electrodes. A Fourier transform is employed to convert it from the time to the frequency domain. The described technologies are combined to produce a variety of different mass spectrometers with diverse properties. As is true for FT-ICR, high-resolution scans require longer recording times. The Orbitrap is sold in combination with a linear ion trap and generates mass spectra with a resolution of up to 100,000 with a mass accuracy of about 1 ppm [19].

11.2.1 On-Line Hyphenation of HPLC to MS

Chromatographic techniques have been used for decades for the separation of highly diversified mixtures. This technique was introduced at the beginning of the twentieth century by the Russian botanist Mikhail Tswett, who separated chlorophylls and carotenoids on calcium carbonate as the stationary phase. One major disadvantage of using open-column chromatography at that time was its high time, sample, stationary-phase, and solvent consumption. Furthermore, the separation efficiency of this technique did not deliver satisfying results. Therefore, HPLC has found its way into laboratories all over the world as a specialized chromatographic tool.

The hyphenation of HPLC to various detection systems has finally led to the breakthrough of HPLC. For the analysis of plant extracts, hyphenation to MS in addition to other detection systems like UV detectors, photodiode array detectors, fluorescence, nuclear magnetic resonance, evaporative light scattering detectors (ELSD), and refractive index detectors is described. The choice of the optimal detection system depends heavily on the class of analytes that are of interest and also on these detectors' varying sensitivities and response to different compound classes. Therefore, in many cases, two or even more detection systems are hyphenated together to simultaneously gather as much information as possible and to clearly elucidate the structures of unknown compounds. One major advantage of MS in contrast to UV, ELSD, or refractive index detection systems is the additional information that can be gained. Besides retention time, information on the molecular mass of detected constituents as well as structural information, given through the generated fragmentation patterns (dependent on the type of mass spectrometer) can be used for identification of detected analytes.

11.2.1.1 Analytical Chromatography—MS

Generally, HPLC-MS systems can be classified into preparative, analytical, micro, nano, and chip HPLC-MS, mainly in the basis of applied column dimensions, flow rates, and injection volumes. Furthermore single-dimensional and multidimensional HPLC-MS can be distinguished with respect to the number of mechanisms involved in the separation process. Table 11.1 gives an overview of the classification of types of HPLC [20].

TABLE 11.1
Inner Diameter of HPLC columns in Relationship to the Classification

HPLC Denotation	Inner Diameter [mm]	Flow Rate
(Semi)Preparative HPLC	>5	>2 mL/min
Conventional HPLC	3.2–4.6	0.5–2.0 mL/min
Microbore HPLC	1.5–3.2	100–500 µL/min
Micro LC	0.5–1.5	1–100 µL/min
Capillary LC	0.15–0.5	1–10 µL/min
Nano LC	0.01–0.15	10–1000 nL/min

The main constituents of an HPLC-MS system are a solvent reservoir, a degassing unit, solvent-delivering pumps, an injection port (auto-injector), a column and a column oven (or chip carrying the stationary phase in direct hyphenation with the ionization unit), an ionization unit, and a mass spectrometer as a detection unit. Furthermore, HPLC can be subdivided into on-line mode (direct hyphenation: ESI, APCI, and APPI), where analytes are detected immediately after elution from the stationary phase, and off-line mode (MALDI), where the measurement of fractionated samples is time-independent from separation.

MS allows an analytical scientist to simultaneously detect and identify dozens of different masses. So the question arises, why is it important to separate the extract before detection? Especially for the qualitative analysis of complex mixtures, a well-separated sample is needed. Although hundreds of components can be detected at the same time through MS in a certain mass range, it is important to separate the extract before detection. Separation of a plant extract allows the identification and discrimination of, for example, stereoisomers and also the detection of analytes present in small quantities. In addition, a well-separated sample minimizes the risk of ionization suppression through a coeluting substance. Next to qualitative concerns, a well-separated sample is indispensable in attempts to quantify target components. Therefore, the choice of applied stationary phase for separation is of utmost importance due to the often-high diversity of a plant extract concerning, for example, the polarity of contained analytes.

One of the most favored HPLC columns in the field of phytochemistry employs modified silica gel C_8, C_{18} (called octadecylsilane [ODS] material), and C_{30} as a stationary phase (reversed-phase chromatography). Carotenoids, considered to help prevent human diseases like cancer and heart disease, were mainly separated via TLC or CC until the establishment of HPLC. The majority of carotenoid separations reported in the literature are carried out on C_{18} and C_{30} columns; the latter ones provide better resolutions especially for compounds with similar polarities [21]. In fact, as shown by our group, especially C_{30} columns benefit on the separation of carotenoids beside also tocopherols. β- and γ-tocopherols are isomers and cannot be separated via a reversed-phase system employing C_{18} stationary phases. Only through use of a C_{30} column and hyphenation to an ion trap mass spectrometer can the nature of analytes be clarified with high reliability, employing reference materials but also unknown biological samples [22]. Flavonoids, consisting of two aromatic and one heterocyclic ring, are universally present in the plant kingdom [23]. Besides occurring in the aglycon form, flavonoids are mostly found as monoglycosides or diglycosides [24]. The separation of these components is mainly based on the use of reversed-phase columns, especially C_{18}. Only through immediate hyphenation to MS, such as to ion trap MS, can the nature of targets be clarified [25].

HPLC systems employing stationary phases with miniaturized particles of 1.7 μm and less are called ultra performance liquid chromatography (UPLC) by Waters Corp. (Milford, United States) or rapid resolution liquid chromatography by Agilent Technologies (Waldbronn, Germany). Stationary phases with particle diameters of 2 μm can theoretically also be used in conventional HPLC systems, but the advantage of shorter separations will be lost due to a lower flow rate required to keep backpressure in an acceptable range. Therefore, suitable equipment, especially regarding pump specifications, has to be used to exploit a UPLC column. Chan et al. described the use of UPLC-TOF-MS for a metabolomic approach to raw and steamed *Panax notoginseng* [26].

Beside modified silica gel particles, a new kind of separation material has been developed over the past years. So-called monolithic packing material finds its application as a stationary phase in conventional HPLC columns as well as in capillaries or chips. The bulk material of such monolithic (continuous-bed) columns consists of a single piece of polymer or cross-linked silica acting as a carrier material for further modifications, as opposed to columns filled with particles [27]. The monolithic polymer possesses interconnected flow paths through the skeleton, which conditions higher column permeability. In this way higher flow rates at lower backpressure and therefore faster separations can be obtained [28]. Monolithic stationary phases can mainly be classified

into organic, inorganic, and mixed forms. As an example of silane-based monolithic stationary phases, silanes like tetramethoxysilane and tetraethoxysilane are polymerized in the presence of polyethylene glycol in a mold or directly in fused silica capillaries. In the work of Tolstikov et al., a C_{18} monolithic silica capillary was applied as the stationary phase in an HPLC–ion trap MS system to evaluate the metabolome of *Arabidopsis thaliana* [29]. Further, Kaufmann et al. used the monolithic column Chromolith (4.6 mm ID × 50 mm) in hyphenation to a single-quadrupole mass spectrometer for the determination of withanolides from *Iochroma gesnerioides* [30]. Especially in this last case, the flow had to be split from 4 mL/min to 400 µL/min, thus conditioning a significant loss of sample.

In addition to the separation of highly diverse extracts through reversed-phase columns, a new approach has to be made to yield a satisfying separation for highly hydrophilic, ionic, and polar molecules, which cannot be done via reversed-phase chromatography. Normal-phase chromatography could form an alternative but is limited due to interfacing problems with ESI, due to potentially occurring difficulties with solubilization of highly polar components in nonpolar solvent systems and due to the diminished ionization efficiency of totally organic and nonpolar eluents in hyphenation to MS [31]. As a response to these problems, the separation using so-called hydrophilic-interaction chromatography (HILIC) was established. In contrast to normal-phase chromatography, HILIC enhances sensitivity in hyphenation to ESI-MS and enables a higher on-column retention of highly hydrophilic, ionic, and polar components [31]. This separation system consists of a polar stationary phase combined with an aqueous and therefore polar organic-solvent mobile phase. In this case water acts as a stronger eluting solvent. In the HILIC mode, the mobile phase consists of 5% to 50% water mixed with acetonitrile or also with acetone–ethyl acetate mixtures. In the beginnings of HILIC, amino silica was employed as a stationary phase to separate carbohydrates [32]. Another kind of stationary phase for HILIC was based on diol and amide silica. The main functional groups, which are nowadays attached to the backbone, are cyano, carbohydrates, diol, and hydroxyl groups [32]. The retention of samples correlates strongly to applied pH and also to the salt concentration.

The measurement of biological samples via HILIC-ESI-MS is documented for oligomeric proanthocyanidins, apple juice, mulberry, and snake venom [31]. Furthermore, in the work of Koh et al., HILIC was employed for the analysis of dencichine in *Panax*, a medicinal plant species used in traditional Chinese medicine. A triple-quadrupole mass spectrometer, which was hyphenated with an ESI source, was employed for this qualitative and quantitative analysis [33]. The use of monolithic HILIC stationary phases has been described for carbohydrates as well [34]. Specifically, oligosaccharides derived from *Arabidopsis thaliana* and soybeans were separated with HPLC-ESI-MS employing a linear ion trap mass spectrometer [34].

A new kind of separation mechanism has recently been introduced into the field of HPLC, although the principle itself had already been used for ages. Especially in the field of large-scale separation of natural products, countercurrent chromatography (CCC) is increasingly used. The separation principle is mainly based on the partition of the analyte between two immiscible liquid phases, such as a mixture of *n*-hexane, *n*-butanol, and water, by vigorous shaking [35]. Furthermore, the target component has to possess different partition coefficients for the mobile as well as for the stationary phase. CCC can mainly be performed with two techniques, coiled tubes and cartridges or disks, differing in their setup. One main advantage of CCC compared to the separation on solid-phase sorbents is the avoidance of the irreversible adsorption of the analyte to the stationary phase [36]. Especially the hyphenation of high-speed CCC (HSCCC) to ESI-MS presents a new and interesting chromatographic tool for identification and fractionation of herbal extracts. In the work of Gutzeit et al., HSCCC was used for the screening and fractionation of polyphenols in buckthorn juice [35]. Another application of CCC in hyphenation with MS was established for separation of flavonoids. Chen et al. optimized separation of three standard flavonoids via HSCCC-ESI-MS and was able to separate and identify flavonoids present in an ethyl acetate extract of *Oroxylum indicum*

[37]. Finally, Ito et al. used CCC directly interfaced with tandem quadrupole MS for the analysis of carbamate pesticides in vegetables and fruits [38].

In addition to single-dimensional HPLC, so-called two- or multidimensional HPLC systems are increasingly used to optimize the separation of rather complex samples of plant extracts. In this case, not just one but two or more stationary phases are involved in the separation process. The idea behind the use of multidimensional HPLC systems is that different classes of chemically similar components are separated on the first stationary phase and then—either on-line immediately after elution of one such class or off-line after fractionation—are separated on a second HPLC column with a different kind of separation mechanism. Various combinations of stationary phases are possible to enhance the separation efficiency. So, for example, a strong cation-exchange column was combined with reversed-phase chromatography in the work done by Pól et al. to characterize *Stevia rebaudiana*. For detection, an ESI-TOF was hyphenated to the HPLC system [39].

11.2.1.2 Miniaturization of Stationary Phases for Direct Coupling to MS

Downsizing of separation columns and chromatographic setups has been a part of the mainstream in HPLC for many years [34–39]. One of the driving forces for the rapid development of microseparation techniques is the growing interest in analyzing minute samples in various fields, such as clinical, pharmaceutical, and forensic chemistry as well as biotechnology. As samples of biological origin usually contain only limited quantities of the analytes of interest, downscaling column LC provides a number of attractive features, namely,

i. An increase in mass sensitivity by the use of concentration-sensitive detectors, allowing further enhancement of the limit of detection (LOD)
ii. Higher chromatographic efficiency in a shorter time period
iii. The ease of on-line hyphenation to MS
iv. Easier control of column temperature due to lower heat capacity
v. Low consumption of mobile as well as stationary phase and thus lower running costs and reduced environmental pollution caused by solvents and chemicals

The improved chromatographic efficiency of microcolumns can be attributed to a decrease in flow dispersion and in the resistance to mass transfer in the mobile phase [37]. In addition, the volume of eluent that is required for elution of the sample compounds is reduced. This leads to an improved signal-to-noise ratio (*S*/*N*), as the volumetric band broadening (dilution) is reduced when using a microcolumn.

The dilution (D) of an injected sample can be calculated using Equation 2.1,

$$\mathrm{D} = \frac{C_{end}}{C_{inj}} = \frac{\varepsilon_{\mathrm{T}} \pi r^2 (1 + \mathrm{k})(2\pi \mathrm{LH})^{\frac{1}{2}}}{V_{inj}}, \tag{11.1}$$

where C_{end} is the concentration of the sample after elution from the column, C_{inj} is the concentration of the sample when it is injected into the column, V_{inj} is the injection volume, ε_{T} is the column porosity, r is the column radius, k' is the retention factor, L is the column length, and H is the plate height. It is apparent that miniaturized HPLC systems have to be designed and operated with utmost attention to eliminating extracolumn band dispersion—attributed to detection volume, sampling volume, and tubing connections—in order to assure high sensitivity and detectability. According to the literature, reducing the column diameter from 4.6 mm to 320 μm results in a substantial increase in peak concentration (~200-fold) and hence detectability [40].

At the same time, miniaturizing HPLC and hence the dimension of the chromatographic column also brings some disadvantages. The flow rate, detection volume, and dead volume as well as the sample volume have to be adjusted with regard to the column dimensions. Therefore, each component of the chromatographic setup has to be modified in order to enable the operation of miniaturized separation columns.

Two different approaches to metabolite analysis on the basis of miniaturized separation systems are being discussed more and more in the scientific community:

- Capillary chromatography employing monolithic stationary phases within the confines of a 200 µm ID fused silica capillary column
- HPLC-Chip on the basis of channels and surface structures on a biocompatible, inert polyimide layer

First, for capillary chromatography, published data prove that monolithic stationary phases show excellent separation efficiency for biopolymers like peptides or proteins. In fact, in 2001 Huber et al. introduced monolithic stationary phases on the basis of poly(styrene-divinylbenzene) for capillary chromatographic separation and consequently direct MS detection of oligonucleotides and proteins [41]. At that time, the limiting factor of analysis was the scan speed of the mass spectrometer, since, owing to the high efficiency of these new stationary phases, not enough time was available to scan the eluting peak and also to perform, for example, fragmentation via MS/MS. Another not-negligible fact is that styrene monoliths seem to be inapplicable for the separation of small molecules, since no publication can be found on this topic. The reason for that lies in the pore-size distribution, as Trojer and Bonn could show a few years later [42].

In fact, the porogen composition, polymerization temperature, and/or initiator content influence the distribution of macropores and thus the separation of analytes of high-molecular weight only. The possibility of controlling the fraction of mesopores, whose distribution is important for successful resolution of small molecules, is therefore conditional [42]. With the publication of this new generation of stationary phases, metabolomics on the basis of capillary LC-MS becomes possible. As an example, the separation of triterpene saponins via HPLC-MS and capillary LC-MS is shown

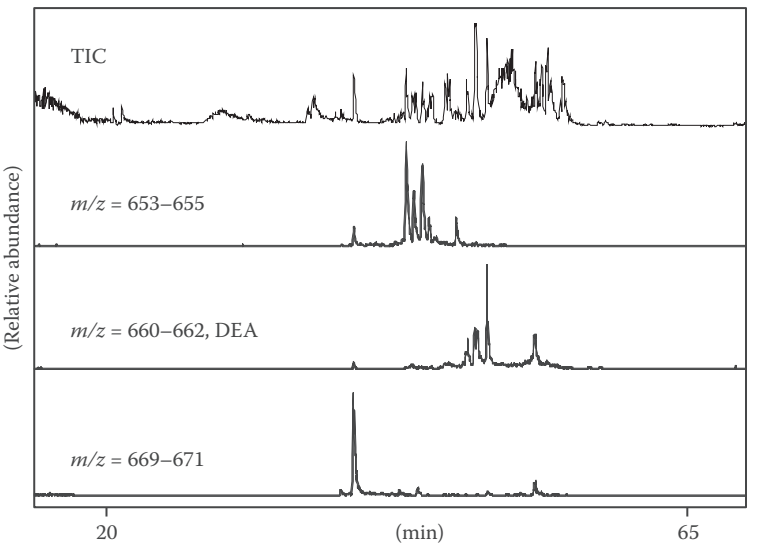

FIGURE 11.5 Separation of triterpene saponins from a *Cimicifuga* extract, employing a polymeric stationary phase according to [42]. TIC, total ion current; DEA, deoxyactein. Depicted *m/z* traces correspond to typically occurring triterpene saponins.

in Figure 11.5. As shown, the former problem of mass spectrometers' limited scan speed has been solved. In fact, new technologies like linear ion traps, as used for the analysis of triterpene saponins, or quadrupole ion traps enable the analysis of signals having only a few seconds of peak width.

The HPLC-Chip was introduced at the AS/MS conference in 2004 and represents the first microfluidic chip-based device that can carry out nanoflow HPLC. The core of this technology is a reusable microfluidic polymer chip, integrating sample enrichment and separation columns. As dead volumes, extracolumn volume, and leak-prone connections do not exist, chromatographic performance is enhanced. Considering also the low-flow rates, it becomes an interesting alternative to HPLC-MS approaches. Chip-LC-MS setups are mainly used for separation and analysis of small amounts of samples. As an example, in the work done by Srbek et al., the protein composition of rice and lentils was determined by use of a chip that was equipped with a reversed-phase column for separation and 5 μm Zorbax material for preconcentration [43]. Further, Buckenmaier et al. published the high sensitivity quantification of pharmaceuticals using Chip-LC with triple-quadrupole MS [44].

11.2.2 Off-Line Hyphenation of HPLC to MS

Within this section a special focus is placed on the use of MALDI-MS as hyphenated MS technique. MALDI-MS is a soft ionization technique employing small, laser-absorbing organic molecules for the ionization and desorption of analytes. The high throughput and sensitivity of the instrument make it an attractive platform for proteomic research. However, the analysis of low-molecular-weight targets is only partially possible. Common matrix substances like cinnamic acid or benzoic acid derivatives cause strong interfering signals below the mass-to-charge ratio (*m*/*z*) of 700. These strong interfering signals can obscure or suppress low-molecular-weight analyte ions. Therefore, typical MALDI spectra are recorded using a proper mass range, such as *m*/*z* 800–3500 for peptides, to avoid disturbing signals and misinterpretation. It means that MALDI is subject to restrictions in the analysis of low-mass analytes due to the codesorption and ionization of matrix molecules during the ionization process. Moreover, the choice of matrix is often critical for optimal desorption and ionization.

In contrast to this disadvantage, MALDI has certain advantages, including tolerance for contamination and buffer, uncomplicated spectra (as most of the analytes are detected as singly charged ions), and very high-absolute sensitivity [45]. Likewise, MALDI allows rapid analysis compared to ESI-MS. These advantages, as well as the demand for high-throughput methods in the areas of drug discovery, biotechnology, and the analysis of complex samples, have reinforced the application of MALDI-MS over the entire mass range. Therefore, interest in analysis of lower-molecular-weight substances has continued to grow in the past decade [46], and several methods have been developed and published during this period [47]: (1) solid (crystalline) organic substances including conventional matrices [48–50], 2,4,6-trihydroxyaceto-phenone (THAP) [51], 3-aminoquinoline [52], and mixtures of matrices [53,54]; (2) solid supports in the form of cobalt

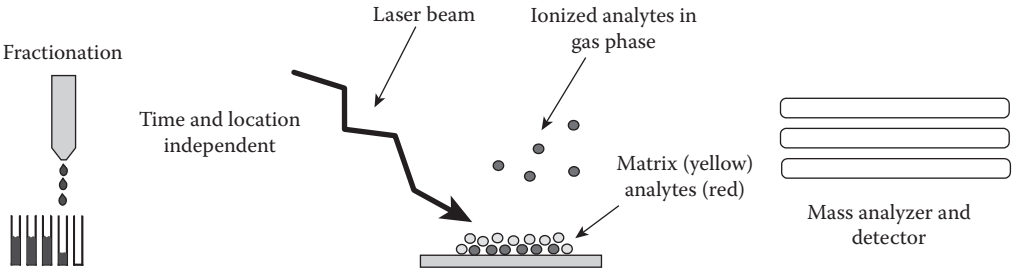

FIGURE 11.6 Matrix-assisted laser desorption ionization (MALDI): Schematic assembling.

powder suspended in glycerol [55], graphite particles [56–58], fullerenes [59,60], porous silicon [61–66], silicon wires [67,68], modified silica gel [62], and carbon nanotubes [69–71]; (3) organic polymers [72–76]; (4) ionic liquid matrices [77,78]; and (5) matrix-suppression effect either in the form of analyte-assisted matrix suppression effect (MSE) [79,80] or with the help of binary mixtures of matrices [81].

In the case of solid (crystalline) organic matrices, considerable background signals may create interferences in the low-mass range and can make the detection or characterization of analytes difficult and therefore complicate the interpretation of spectra. Somehow few organic crystalline matrices have been employed in certain cases to characterize low-molecular-weight samples [82]. An alternative approach to organic crystalline matrices, called inorganic matrices or solid support, has been adopted to address this problem. Tanaka et al. originally introduced this technique in 1988, employing cobalt powder suspension in glycerol [55]. Afterward, Sunner et al. developed the surface-assisted laser desorption/ionization (SALDI) technique using graphite particles suspended in various solvents or a mixture of solvents for the analysis of organic molecules and peptides [56]. Subsequently, carbon nanotubes in the form of immobilized carbon nanotubes [69] and oxidized carbon nanotubes [70,71] have been found to provide even better results than SALDI. Wei et al. introduced the use of electrochemically etched porous silicon for the first time for desorption and ionization of low-molecular-weight analytes [61]. In addition, some other silica-based matrix-free approaches, the use of porous silicon [62–66], nano silicon wires [67,68], and modified silica gel [62], were employed for the analysis of low-molecular-weight analytes.

Concerning the application of organic polymers as MALDI matrices, Ayorinde et al. and Jones et al. screened some porphyrin derivatives [75,76] in order to circumvent the interference in the low-molecular-mass range. Liquid matrices were developed in order to improve the signal reproducibility from shot-to-shot and sample-to-sample, but these suffer from high-chemical background, potential contamination of the instrument, and poor ionization efficiency [83]. Some other techniques like analyte-assisted matrix suppression [79,80] and binary mixtures of matrices or additives [53,81,84] have also been determined to accomplish the desired results for the analysis of low-molecular-weight analytes. Chan et al. [79] introduced the matrix-ion-suppression model for the first time in 1991. This analyte-assisted matrix-suppression approach was based on a competition model for protons, in which the matrix protons (primary proton carrier) compete with analytes (proton acceptor). According to the model, every primary proton carrier interacts with at least one analyte, which has the ability to take a proton and can be detected as a protonated analyte in MALDI-MS. This means that an analyte with a high-proton affinity and in high-enough concentration may suppress all other possible protonated-ion signals.

Nevertheless, Chan's proton competition model suffers many limitations [79,85]: First, the analysis of those analytes that are detected as alkali metal adducts cannot be explained by means of this model, and, second, the analyte-assisted matrix-suppression approach always requires the correct ratio of analyte to matrix to efficiently suppress the background signals produced by the matrix. This last point, in particular, always implies a high concentration of analytes. Furthermore, the method of analyte-assisted matrix suppression is not effective for carbohydrates due to the non-availability of basic sites, which are necessary for the acceptance of protons to suppress the matrix background signals. As a result, strong and too intense matrix background signals and less intense analyte signals are obtained.

However, the main point is that desorption, ionization, detection of particular analytes, and matrix-suppression effects depend mainly on the selection of matrix materials and the nature of the analytes. Furthermore, binary mixtures of matrices or additives have been evaluated in order to upgrade the crystal formation within the analyte-matrix system. The aim was a more homogenous embedding of analytes throughout the target spot, finally enhancing the signal intensities of analytes, and at the same time also improving mass resolution and reproducibility [53,86,87]. Published data utilizing binary mixtures and additives still highlight the problems below *m/z* 500 with intense signals appearing up to *m/z* 350 [53]. In this context, in 2008 we published new matrix combinations,

employing mixtures of different acids and bases. In particular for the analysis of carbohydrates, we could show the excellent performance of dihydroxybenzoic acid and aminopyrazine [88].

In 2005 our group introduced the material-enhanced laser desorption ionization (MELDI) approach. This approach utilizes chromatographic materials with their morphological parameters like porosity, particle size, and surface area alongside the chemical interactions of the affinity materials with the analyte. Each parameter has its own importance and impact on the MELDI process and therefore conditions the optimization of the system. For example, spherical cellulose with a particle size of 8–10 µm performs excellently in protein profiling [89], while highly porous silica gel has to be optimized concerning particle size (300 and 1000 Å) [90]. Additionally, the material-characteristics effects like hydrophilicity and hydrophobicity can also be observed over the mass fingerprints [89]. A large number of material-based techniques have been applied for a variety of purposes using surface modifications of MELDI materials (Figure 11.7). The employed MELDI materials include cellulose [89,91], silica gel [89,90], polymers [89,93,94], diamond-like carbon [89,94,95], carbon nanotubes [95,96], nanofibers, and C60 fullerenes [95,97–99]. The main application field of MELDI is clinical proteomics [100]. The advantages of MELDI are the improvement in sensitivity, specificity, loading capacity, and reproducibility alongside the availability of more surface area and number of masses detected compared to other techniques in this field.

The application of MELDI-MS for the analysis of primary and secondary plant metabolites has been described in recent publications [101–103]. By immobilizing 4,4'-azodianile on silica particles, a well-developed aromatic conjugated system, with an absorption maximum at approximately 330 nm, is available. This delivers the basis for a successful desorption and ionization process, and consequently also improves the signal intensity of the target compounds, as no background noise is detected. It is well known that effective MALDI matrices have an aromatic conjugated system, which lowers the overall energy of the molecule and thereby increases its stability. By fixing the aromatic energy-absorbing unit via a covalent bond on the carrier system, background signals and noise are avoided, and analysis of small molecules becomes possible. In exceptional cases, such covalent-bound systems consist of instable spacers, which can be used for the immobilization of macromolecules, to study surface-based photochemical-reaction mechanisms (surface-enhanced photolabile attachment and release) [104].

The analysis of biological samples such as *Salix alba* L., *Taxus baccata* L., *Pinus cembra* L., *Betula pendula* Roth., *Picea abies* L., and *Quercus robur* plant extracts and xylem saps, as well as wheat straw-degradation products (hydrolysis degradation, Aquasolv degradation) was published in this context [101,102].

As an example, a *Taxus baccata* water–methanol extract spectrum is depicted in Figure 11.8. In addition to taxol (or paclitaxel) its precursor ions were also of main interest, such as 10-deacetylbaccatin, cephalomannin, and baccatin III, as those substances can be isolated from needles of the plant and derivatized in vitro into the pharmaceutically needed paclitaxel. The analysis of freshly prepared raw extract showed a clear sodium signal for 10-deacetlybaccatin.

11.3 APPLICATIONS

11.3.1 Structural Elucidation of Analytes

11.3.1.1 *C*- and *O*-Glycosilation of Flavonoids

As an example of the identification of metabolites in plant samples, the group of *O*- and/or *C*-glycosilated flavonoids is considered here. Flavonoids belong to the group of secondary plant metabolites with manifold structures. They are widespread in plants and are of significant therapeutic importance. Flavonoids consist of a three-ring diphenylpropane ($C_6C_3C_6$) core with hydroxylation and/or methylation at positions C-3, C-5, C-7, C-3′, C-4′, and C-5′. Additional modifications with aromatic or aliphatic acids and sulphate, prenyl, or isoprenyl groups are possible, although the most frequent modifications are *O*- and *C*-glycosides. In case of *O*-glycosides the aglycone (the

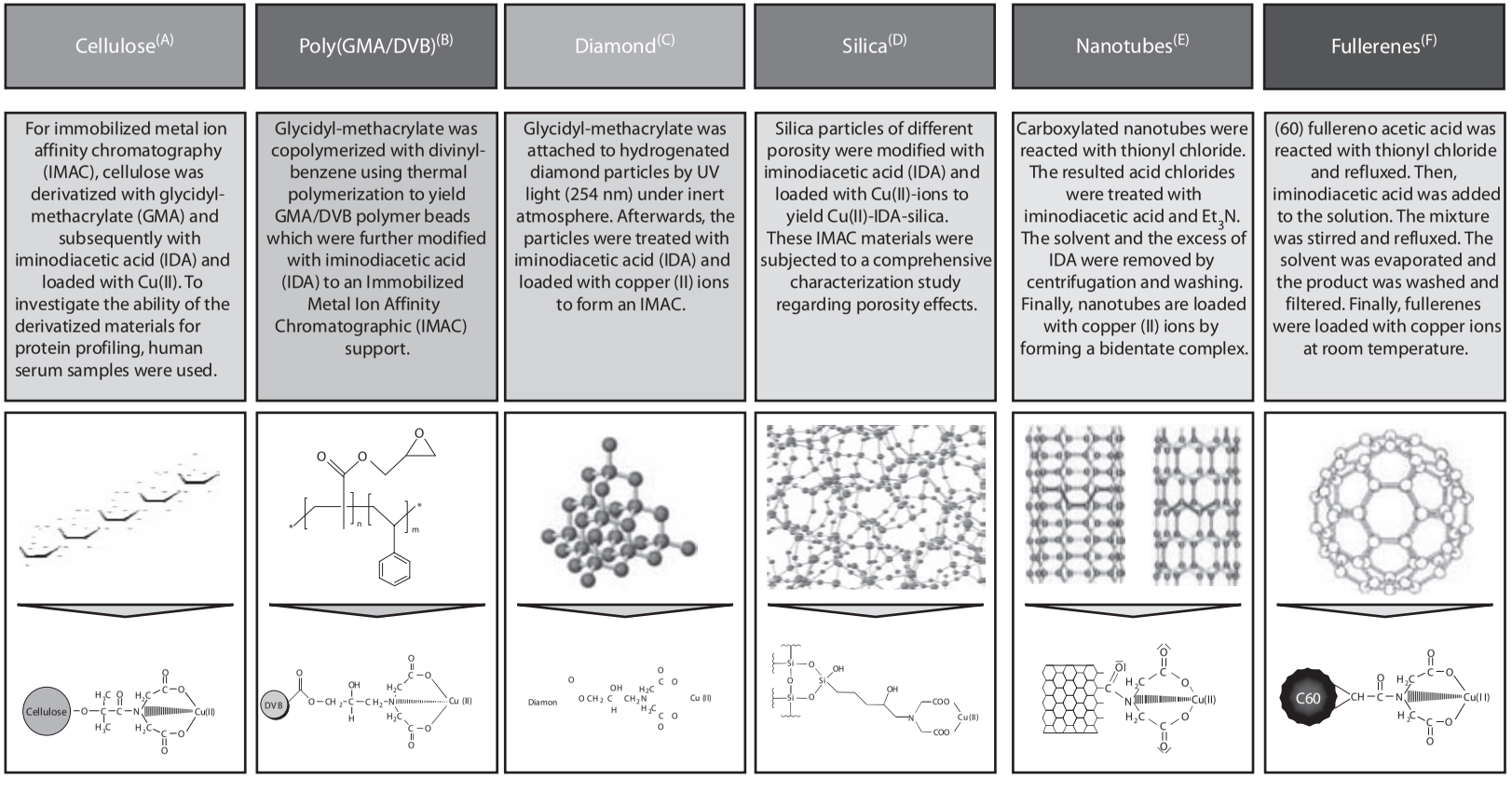

FIGURE 11.7 An overview of different material enhanced laser desorption ionization (MELDI) supports. A = [89,91], B = [89,92,93], C = [89,94,95], D = [89,90], E = [95,96], and F = [95,97,99].

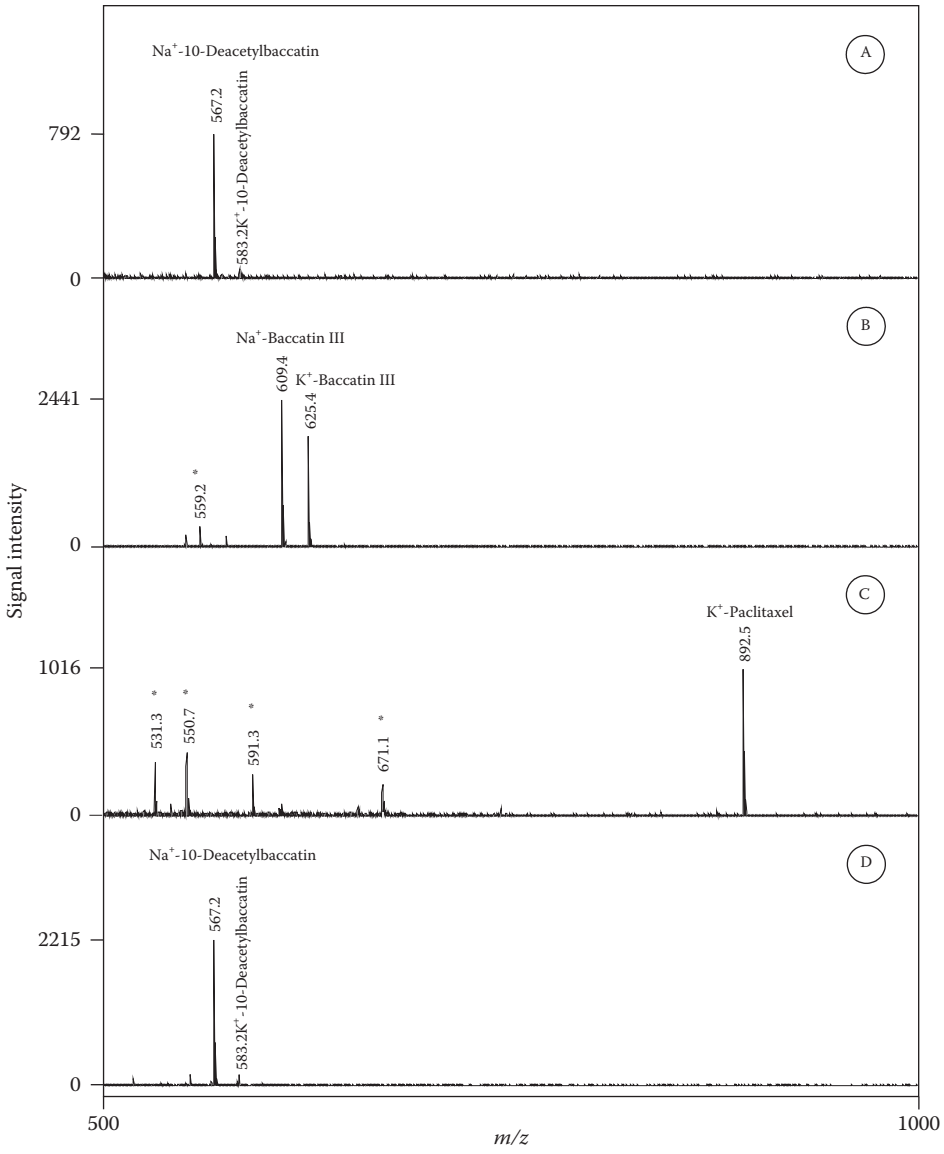

FIGURE 11.8 Matrix-free material enhanced laser desorption ionization mass spectrometry (MELDI-MS) spectrum of *Taxus* standards and plant extract using azodianiline modified silica; (A) deacetylbaccatin III (standard), (B) baccatin III (standard), (C) paclitaxel, and (D) purified *Taxus baccata* extract. Asterisks represent unidentified signals.

unmodified core structure) is linked to mostly one or two substituents via a hydroxyl group, while *C*-glycosides are attached via a carbon–carbon bond. The most frequently observed monosaccharide units are glucose and rhamnose, but arabinose, xylose, and glucuronic acid also occur.

For the fragments produced by MS^n measurements, a widely accepted nomenclature is used. The system for aglycones was developed by Mabry et al. [105] and further improved by Ma et al. [106]. Elaboration for glycoconjugates was done by Domon and Costello [107]. In the following, the fragmentation products and their identification by MS^n are discussed briefly. For the fragmentation of ions generally, the method of ionization, the type of mass spectrometer (in

some cases even the manufacturer), and the fragmentation process (collision-induced dissociation, electron capture dissociation, electron transfer dissociation) have to be considered and may lead to the occurrence of different fragment masses and intensities. However, flavonoids are reported to show very reproducible main fragmentation paths, even under different ionization methods and mass analyzers. The emphasis lies on the expression "main fragmentation paths," as the relative abundance of observed fragment ions change significantly between measurements with different instrumentation. Therefore, the interpretation of spectra should be based on the presence and not on the relative abundance of fragment ions if using data from the literature or from other mass spectrometers. For the differentiation of isomers, comparative studies of known standards might be indispensable. Depending on the structures investigated, positive or negative ionization modes, or often both, are necessary to generate sufficient information for unambiguous identification.

A first step in identifying a flavonoid is the differentiation between *O*-, *C*-, and *C*,*O*-glycosides, which can be done in both positive and negative ionization, although these produce different intensities and also partial different fragments. Although flavonoid glycans with three or more sugars attached are known, hardly any information about their identification has been published. The fragment spectra of flavonoid glycosides do not provide information about the stereochemistry of the glycan, but the available information allows clear differentiation between hexoses, deoxyhexoses, and pentoses. For *O*-glycosides, low to medium fragmentation energy is applied to cleave the relatively weak hemi-acetal C-O bond, producing neutral loss peaks that allow the mass of the attached sugars to be calculated. If fragmentation is performed at higher energy, additional fragmentation occurs, complicating the interpretation. For example the loss of -146, -162, and -308 Da indicates the presence of a hexose and a deoxyhexose unit on the flavonoid. The neutral loss fragment of -308 provides initial information about the aglycone. On the other hand, the bond of the sugars of *C*-glycosides with the C-C bond is stronger than the C-O bond of *O*-glycosides, though higher fragmentation energy has to be applied to cleave the sugar ring's C-C bond. Consequently, the sugar moiety itself is cleaved intraglycosidic. For example, from the losses of 120 and 122 Da, the presence of a hexose and a deoxyhexose can be deduced.

Flavonoid *O*-glycosides containing two monosaccharides can have the structure of di-*O*-glycosides or di-*C*,*O*-glycosides, in case of an attached disaccharide *O*-diglycoside or *C*,*O*-diglycoside. Differentiation is possible by analyzing the MS/MS fragment spectra in positive and negative ionization modes for the presence of indicative fragments and relative abundances. The characterization of flavonoid *C*-glycosides is trickier. The assignment of the sugar units of di-*C*-glycoside seems to be possible in only some cases. The mapping of *C*,*O*-flavonoid binding types can be achieved only by employing standards.

For the identification of the aglycone, initial information is given by the occurring fragment of the MS/MS spectra, indicating the basic structure and the number and nature of substituents. More, and also more precise information is provided by the generation of MS^3 spectra of the aglycone in the case of flavonoid *O*-glycosides or the aglycone with the sugar rests attached. From the generated fragments, the identification of the whole structure is possible.

In summary, from MS^n measurements, the following information about flavonoids can be gained:

- Glycosylation type (*O*-, *C*-, or mixed glycosides)
- Aglycon identity
- Types of carbohydrates
- Sequence of the glycan part
- Interglycosidic linkages
- Binding sites of the sugars to the aglycone

11.3.1.2 Triterpene Saponins

The importance of MS analysis of plant samples, especially of triterpene saponins, is elucidated here via the analysis of black cohosh, that is, *Cimicifuga racemosa*. Triterpene glycosides, phenolic compounds, and carbohydrates are the main compounds present in *Cimicifuga racemosa* rhizomes. To date, as summarized by Nuntanakorn et al. [109], over 40 triterpenoid glycosides have been isolated, which exhibit various biological activities, including anti-cancer activity, anti-HIV activity, and an inhibitory effect on catecholamine secretion. Further, over 13 polyphenolic compounds have been isolated and described, including hydroxycinnamic acid derivatives, fukiic acid ester derivatives, and piscidic acid ester derivatives.

Generally, newer publications focus on the isolation and characterization of new triterpene glycosides or phenolic compounds [110–114]. Published quantified amounts refer to total triterpene saponin content, since for single analytes reference standards are hardly available. Jiang et al. [115] published a total amount of 0.5–10.5% of seven triterpene saponins and 0.5–6.6% for six phenolic constituents. Panossian et al. found 2.11% triterpene glycosides in the roots of black cohosh [116].

The results from a mass fingerprint study of *Cimicifuga racemosa* plant extract via MELDI-MS is shown in the following: Extracting *Cimicifuga racemosa* with different extraction solvents like water, water in acidic media, water in basic media, methanol, 50% methanol, ethanol, 50% ethanol, acetone, and others delivered dominant carbohydrate signals for the water extract and dominant signals for triterpenes for the acetone fraction. The study revealed the presence of sugars in the sample. At the same time, many signals were also observed within the mass range $m/z = 600–750$. These resulted from triterpene glycosides.

A big problem related to triterpene saponins is their large heterogeneity, and, especially in MS, their strong tendency to produce fragments during the ionization process. In order to enhance data evaluation, a comparison with commercially available reference materials is extremely important to recognize the typical behavior of this substance group. For LC-MS investigations of triterpene saponins, the loss of an acetic acid function (–60 Da) and the loss of a sugar moiety (e.g., xylose; -132 Da) is typical. From the intact molecule and from different main fragments, between one and three units of water can further be split off.

A very similar behavior is noticeable in MELDI-MS with the addition of the detection of quasi-molecular ions in the form of sodium and potassium adducts. In fact, actein and 27-deoxyactyein were also detected as their sodium and potassium adducts at *m/z* 699.1, 715.1, 683.1, and 699.1 respectively. Fragments of actein were found at *m/z* 639, 467, and 449 and assigned to $(MNa\text{-}HCO_2CH_3)^+$, $(MH\text{-}HCO_2CH_3\text{-xyl-}H_2O)^+$, and $(MH\text{-}HCO_2CH_3\text{-xyl-}2H_2O)^+$, respectively. The fragments arose at the same or with slightly higher intensity than the molecular ions. Signals at *m*/z 674 and 647 could not be allocated with published data. Some other signals with *m/z* values of 240, 308, 238, and 416 were detected within the range *m/z* 220–420 that could not be interpreted. In the case of deoxyactein, a fragment at *m/z* 623 was detected and assigned to $(MNa\text{-}HCO_2CH_3)^+$. The investigation of the single standards actein and deoxyactein with mf-MELDI-MS showed the presence of sodium and potassium adducts, but the protonated peak could not be found within both analytes.

Therefore, the resulting spectra (Figures 11.9 and 11.10) of matrix free (mf) MELDI-MS measurements of *Cimicifuga racemosa* extracts were examined on the basis of the presence of their sodium and potassium adducts for triterpene glycosides. For *Cimicifuga racemosa*, 15 root triterpene glycosides could be assigned via MELDI-MS, matching data with the literature [110,117–120].

11.3.2 Quantification of Selected Analytes via LC-MS and MALDI-MS

MS can be employed as quantification tool for low-molecular-weight analytes. Nevertheless, not every type of instrument is suitable, as the geometry of the instrument, like ion-transfer optics and the mass analyzer, has a strong influence on reproducible availability of ions. Therefore, for

quantitative LC-MS approaches, single- and triple-quadrupole instruments are preferred, as these types of instruments generate quantified data with a low-variation coefficient. In contrast, the aim of ion trap mass spectrometers is the trapping of ions and the production of fragmentation patterns. Trapping time strongly influences the availability of ions and leads to a coefficient of variation for 3D traps of approximately 15%. TOF mass spectrometers also suffer from unreproducible availability of ions. In fact, MALDI-TOF-MS and HPLC-Q-TOF-MS both suffer from strong variations concerning quantification. In the case of MALDI-TOF-MS, the MALDI process itself is responsible for this fact, as ions are embedded within the matrix. Through the application of laser energy, analytes are released by the matrix. The number of analytes depends heavily on the local area where the laser is focusing. Therefore, spot-to-spot reproducibility is very low. In the case of HPLC-Q-TOF-MS, variation is reduced but still not satisfying, as ion packages are pulled into the TOF through voltage pulses applied after the quadrupole mass filter.

MALDI-MS will be focused stronger, as it enables a fully automated screening of samples within a very short time. In fact, in proteomics, it is used as a routine screening platform. The limitations and newest development of MALDI in the direction of MELDI has already been described and discussed. Concerning the quantification of low-molecular-weight analytes via MALDI-MS, Cohen and Gusev [83] review the state of the art and conclude that the mass resolution and linearity of acquisition systems had been one of the major obstacles in early applications of MALDI. Developments like delayed extraction, powerful ion detectors, and high-speed acquisition systems have adequately addressed most of the instrumental issues, but the main limiting factor is still intra (sample-to-sample) and inter (point-to-point and shot-to-shot) reproducibility. One fundamental point found in a variety of publications is the use of an internal standard. In the optimal case this standard represents a labeled isotope of the substance of interest, as in this way, for example, ionizing behavior is considered and reproducibility is increased. A variety of quantification approaches are summarized by Cohen und Gusev [83].

11.3.3 Metabolomics

In the years when genomics entered the mainstream of our society as a new magic bullet in the fight against diseases as well as a hope to get a step forward in the discovery of fountain of youth, another -omic project, metabolomics, began to establish itself in the shadow of its superior brother. After a period of disillusion in the -omics sciences, it became obvious that the circumstances of interactions and procedures taking place in a living organism were even more complex as they had been thought to be.

Metabolomics can be described as an approach of thoroughly analyzing the metabolites present in a target organism, specific cells, or tissues, while metabolites are characterized as educts or products of various metabolic reactions taking place in a cell. Furthermore, primary and secondary metabolites can be distinguished. Carbohydrates, amino acids, fatty acids, and organic acids belong to the class of primary metabolites that are involved in the growth and development of plants as well as in respiration, photosynthesis, and hormone and protein synthesis [40]. Secondary metabolites are components like flavonoids, sterols, carotenoids, phenolic acids, and alkaloids, which protect plants against herbivores and microorganisms. Furthermore, they play a key role in reproduction, for example, by attracting pollinators. Due to hundreds and even thousands of metabolites, it is nearly impossible to describe all ongoing reactions and metabolisms in a cell. The plant kingdom has an estimated 200,000 primary and secondary metabolites, whereas the human metabolome is probably even larger [121].

The complexity of metabolomics studies has led to a subdivision into subareas like target-component analysis (qualitative and quantitative analysis of one or only a few metabolites representing a certain reaction), metabolite profiling (identification and quantification of certain metabolites present in a specific metabolic pathway), metabolomics (qualitative and quantitative analysis of all metabolites), and metabolic fingerprinting (fast and global analysis of samples to afford sample classification), according to a proposal by Fiehn [121,122]. Furthermore, metabonomics deals with

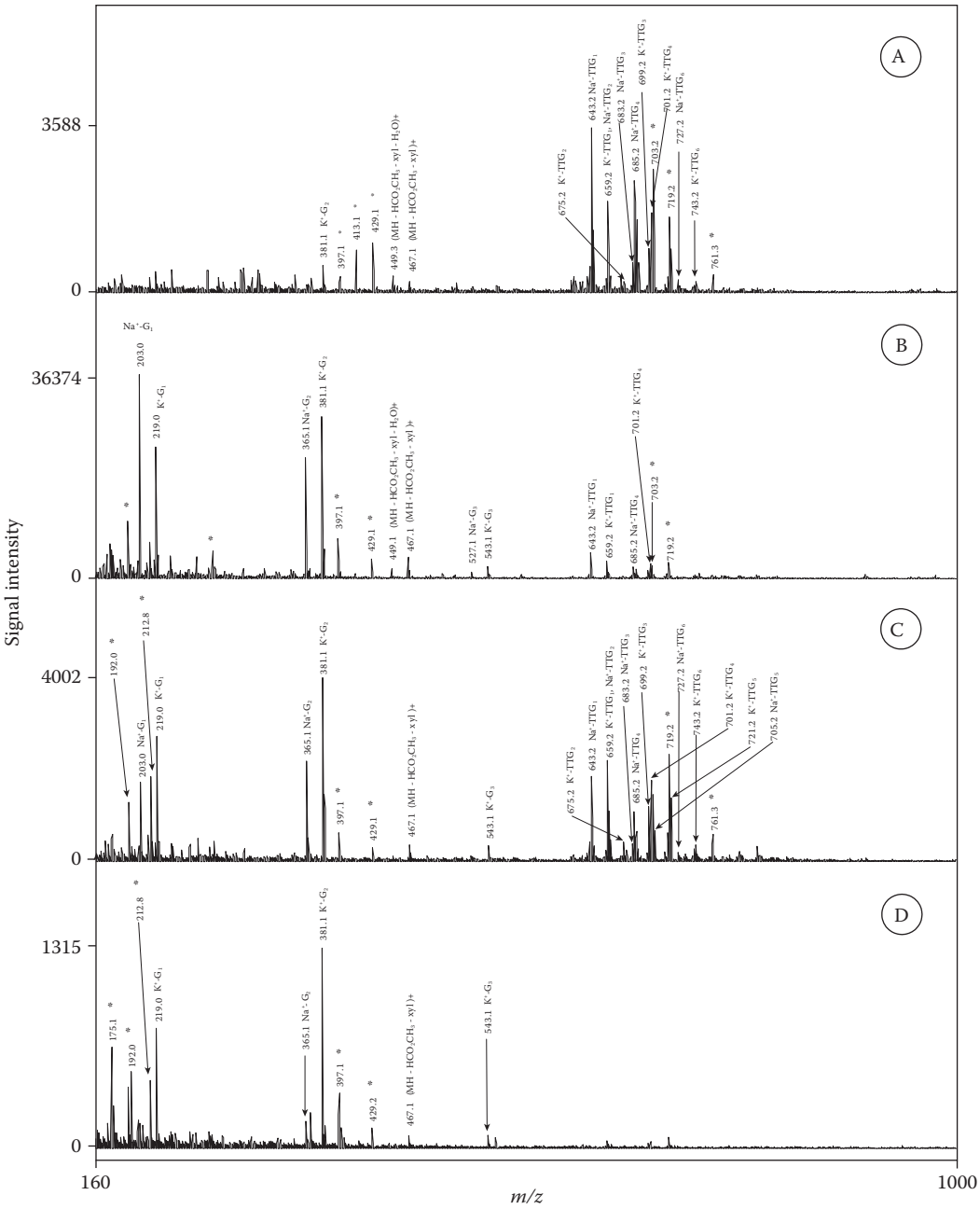

FIGURE 11.9 Matrix-free material enhanced laser desorption ionization mass spectrometry (MELDI-MS) spectrum of *Cimicifuga racemosa* in different extraction conditions using azodianiline modified silica; (A) water extract, (B) methanol extract, (C) ethanol extract, and (D) extraction at pH 2. G_1, glucose, fructose; G_2, sucrose; G_3, raffinose; TTG, triterpene glycosides. The rest of the peaks are labeled with the corresponding names of analytes. Asterisks represent unidentified peaks. Each spectrum corresponds to 500 laser shots.

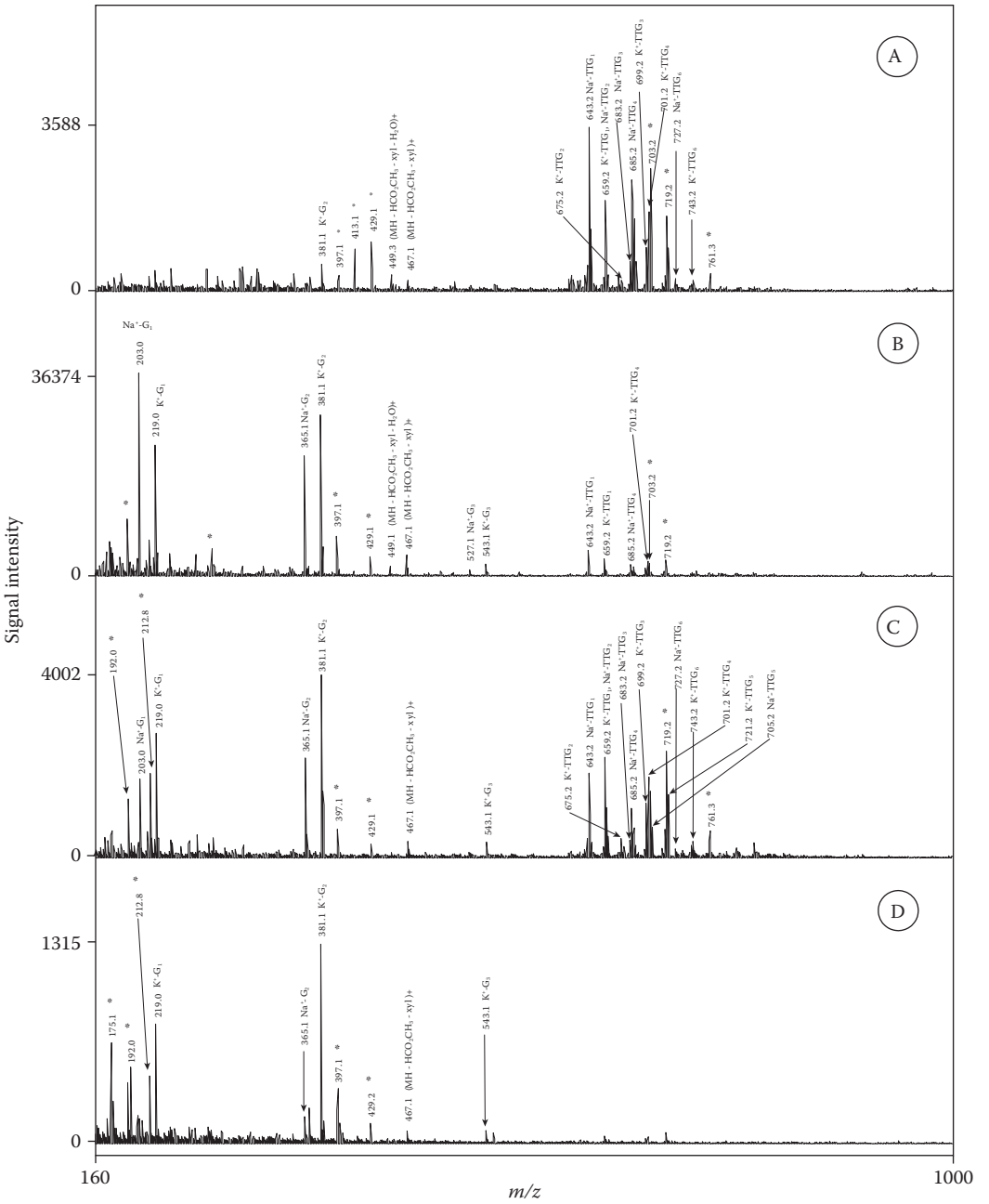

FIGURE 11.10 Matrix-free MELDI-MS spectrum of *Cimicifuga racemosa* using azodianiline modified silica; (A) acetone extract, (B) 50% ethanol/water extract, (C) 50% methanol–water extract, and (D) extraction at pH 10. G_1, glucose, fructose; G_2, sucrose; G_3, raffinose; TTG, triterpeneglycosides. The rest of the peaks are labeled with the corresponding names of analytes. Asterisks represent unidentified peaks. Each spectrum corresponds to 500 laser shots.

the evaluation of tissues and biological fluids for fluctuations in metabolite levels that are caused by diseases or therapeutic treatments [121].

On closer inspection of plant metabolites, they will very probably turn out to be a valuable source for the development of new medicine [123]. Among newly developed drugs introduced onto the market in recent years, the majority of active components were based on derivatives of metabolites or even unmodified plant constituents. Cancer chemotherapeutic drugs like paclitaxel (taxol) and podophyllotoxins (etoposide, teniposide) have their origin in secondary plant metabolites [123]. As a reaction to environmental influences, a huge arsenal of secondary plant metabolites was synthesized for acquiring some defense strategies, which puts them into the spotlight for the discovery of active substances. In regard to an urgent demand for newly developed and introduced antibiotics, the search for further active substances has broadened into the plant kingdom. The development of multidrug-resistant bacterial strains accompanied by decreased inhibition potency of antibiotics increasingly complicates modern forms of clinical therapies. Even drugs like vancomycin used as last bastions in the treatment of multiple-resistant bacterial infections are becoming more often ineffective [124]. Notably, the number of infections acquired in hospitals (nosocomial infections) are alarming. About 1.7 million infections and 99,000 cases of death are registered just in the United States per year [125,126]. In particular, the extensive use of antibiotics beyond clinical therapy in agriculture and ordinary household articles contributes to the ineffectiveness of several antibacterial medications. These reasons make investigations into plant constituents more interesting despite problems that occur in regard to identification strategies, stability, and availability.

For investigations on a metabolome, MS is one widely used technique due to its blend of rapid, selective quantitative and qualitative analysis for identification of metabolites as well as its sensitivity. Hyphenated techniques like gas chromatography-MS and HPLC-MS are often used [121]. Due to its restriction to volatile and thermally stable components, different kinds of derivatiation methodologies are applied for low-weight metabolites for analysis via gas chromatography–MS.

The absence of suitable databases with already recorded metabolites makes the identification of unknown components more difficult in the case of HPLC-MS, in contrast to gas chromatography–MS. To gather some information on fluctuations in the content of different plant metabolites, which are up- or down-regulated due to, for example, environmental influences, quantitation of selected metabolites is essential. For gathering reproducible and interpretable results, precautions have to be made for the quantitative approach. Due to the influence of ionization effects as well as the applied type of mass spectrometer, quantitative analysis can result in inaccurate conclusions, especially in case of hyphenated techniques like HPLC-MS.

Concerning HPLC-MS especially, triple-quadrupole mass spectrometers are employed for screening high numbers of samples. In fact, tandem MS allows, in addition to the full scan analysis, proof of identity via multiple reaction monitoring analysis or special investigative approaches like parent ion scan or neutral loss. On the other hand, high-resolution mass spectrometers like Q-TOF mass spectrometers are essential for the crucial investigation field of glycomics and of glycoproteomics, two crucial points in phytochemical metabolomics.

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12 LC-NMR and Related Techniques for the Rapid Identification of Plant Metabolites

Jean-Luc Wolfender

CONTENTS

12.1 NMR AS A DETECTOR FOR HPLC

The improvements in sensitivity and resolution recorded in nuclear magnetic resonance (NMR) methods over the last years have strongly accelerated the pace at which natural products (NPs) are identified. Within these different technological advances, modifications in magnet technology and electronics have led to a smooth but steady development over the years, while the application of more sensitive probe heads such as microcoils or cryogenically cooled probe heads and the application of pulsed field gradients have resulted in more dramatic changes [1].

In this respect, the coupling of high performance liquid chromatography (HPLC) with NMR spectroscopy (LC-NMR) represents one of the most powerful methods for the separation and structural elucidation of NPs in mixtures [2].

From a historical viewpoint, the interest in combining separation methods with ^{1}H-NMR spectroscopy arose in the late 1970s [3–6]. The inherent lack of sensitivity of NMR instruments at that time, and the problems related to efficient solvent suppression, delayed the beginning of practical use of LC-NMR to solve analytical problems by almost two decades [7].

Since the mid-1990s, LC-NMR has been established as a very efficient method for the on-line identification of organic molecules, and it was readily applied to the structural identification of NPs in crude plant extracts. From this time, new developments of efficient analytical strategies, which comprise both direct and indirect hyphenation of NMR with HPLC, have been proposed. In the light of the needs in phytochemical analysis and plant metabolomics for both on-line identification and dereplication purposes, the possibilities and limitations of these approaches are discussed here.

12.1.1 Principle of LC-NMR

LC-NMR basically results from the coupling of HPLC, which is known to be the most versatile technique for the efficient separation of secondary metabolites directly in the crude mixture, with NMR, the detection method that provides a richer type of structural information on separated analytes. The application of NMR as an HPLC detector can thus be regarded as an ideal combination for both the separation and structural identification of NPs. Furthermore, the advantage of LC-NMR resides not only in the fact that full structural and stereochemical information can be obtained (by use of 2D NMR), but also in it being a highly nonselective detection technique (^{1}H-NMR spectroscopy will detect any hydrogen-containing compound present in the HPLC eluate in a sufficient amount regardless of its structure) [7,8]. LC-NMR should thus ideally enable the complete structural characterization of any plant metabolite directly in an extract, provided that its corresponding LC peak is clearly resolved. In practice, however, this statement is not entirely true, as many factors hinder on-line structure determination [9]. These problems are mainly linked to the inherent low sensitivity of NMR for the detection of microgram or submicrogram quantities of NPs separated by conventional HPLC, and the need for solvent suppression.

Indeed, LC-NMR can be regarded as relatively insensitive compared to other detection methods, such as LC–photodiode array detector (DAD)–ultraviolet (UV) or LC–mass spectrometry (MS), due to the intrinsic properties of NMR [8]. These limits also depend on the type of magnet used (400–900 MHz) and are strongly related to the type of flow probe used and the mode of operation (direct or indirect hyphenation, at-line use).

12.1.2 Flow Cell NMR Probes

The heart of an LC-NMR system includes a dedicated flow probe through which the eluant from the HPLC has to flow and carry the separated analytes. The design of such continuous-flow NMR probes, introduced in the early 1980s, was a contradiction to the classical conventional tubes setup. In this design, a U-type glass tube was fixed in the Dewar of the NMR probe body, breaking the central symmetry of the magnetic field. The first application of this flow cell ("saddle"-shaped geometry; Figure 12.1f) showed excellent resolution, approaching those registered with rotation of the NMR tube [10]. This type of cell employs detection volumes between 40 and 120 µL, which are much larger than conventional UV detection volumes (8 µL) [2]. The use of such significant detection volumes resides on a required compromise between the needs of chromatography and those of NMR. Indeed, while conventional HPLC can accommodate detection volumes of 5 to 10 µL, NMR needs extremely large detection volumes for line-shape quality and sensitivity. The line shape in the on-flow mode is, indeed, directly related to the residence time of the analyte in the flow cell, and the measured signal half width increases with increasing flow rate [2,11]. LC-NMR

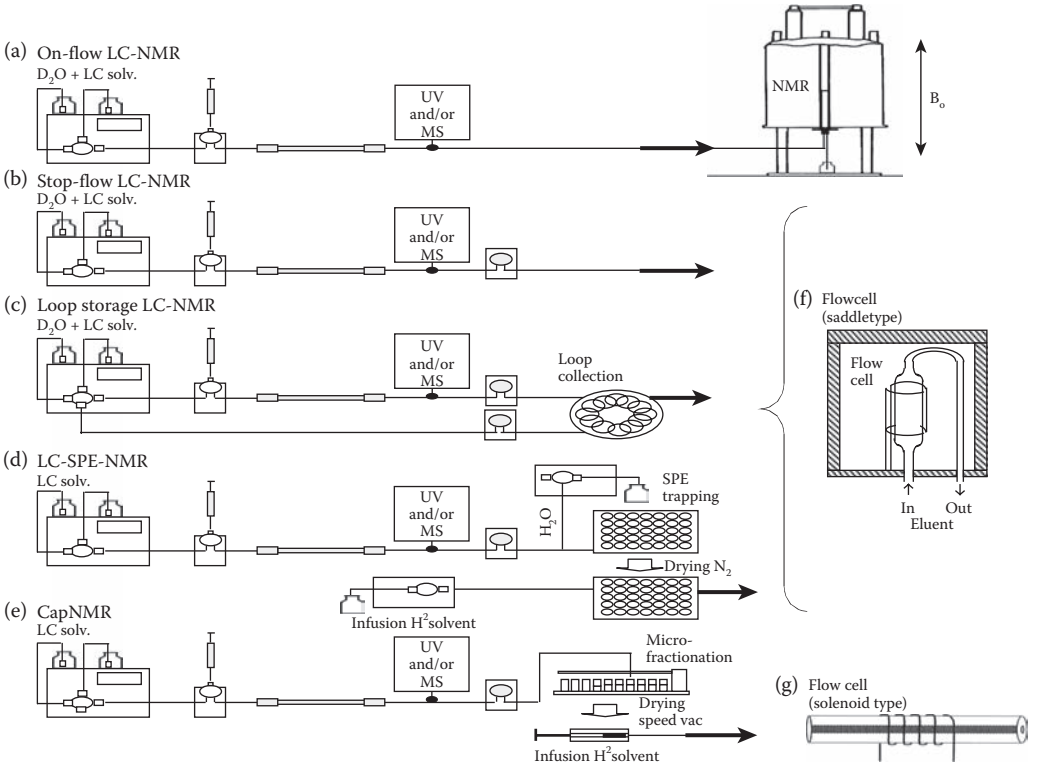

FIGURE 12.1 Various modes of operation of liquid chromatography–nuclear magnetic resonance (LC-NMR) in direct (a–c) and indirect (d and e) hyphenation. Schematics of saddle-type continuous-flow LC-NMR probes (f) for the operation modes a–d and of solenoidal continuous-flow LC-NMR probes (g) (for operation with capillary NMR [capillary NMR] and microfractionation).

is a volume-sensitive detection technique and requires the maximization of NMR-active nuclei by extension of the detection volume. An optimum detection volume cannot be found for all LC peaks during elution. Indeed, NMR detection of the dilute solutions in later LC peaks would require very high detection volumes in the mL range, due to the diffusion of the LC chromatographic process.

The design of the actual flow cells is thus the result of a compromise between the needs for resolution and sensitivity on the NMR side (large volume) and reasonable LC peak separation on the chromatographic side (small volume). As is discussed later, a lesser compromise must be made in the indirect hyphenation of NMR. In this case, the LC peaks can be preconcentrated on solid-phase extraction (SPE) or by drying and redilution in deuterated solvents, and their elution volume optimized to match that of the flow probe. Regular LC-NMR flow probes (30–60 μL) or microflow probes with an extremely small active volume (5 μL) can be employed.

In this respect, reduced-diameter solenoid NMR coils (capillary NMR, or CapNMR) represent a particularly attractive approach to enhancing NMR sensitivity for small-volume, mass-limited sample uses (Figure 12.1g) [12]. As predicted theoretically and verified experimentally, the mass sensitivity, defined as signal-to-noise (S/N) per sample quantity, of an NMR coil depends inversely on its diameter [13]. However, for a given coil diameter, solenoidal coils exhibit a several-fold enhancement in NMR sensitivity, compared with that of Helmholtz coils. Because of the different advantages afforded by the two coil geometries, the specific application determines the best choice for the LC-NMR probe heads (standard LC-NMR probes 40–120 μL, microcoils 0.02–5 μL or cryo-flow probes).

The mode of operation also strongly affects sensitivity. Indeed, for on-flow measurements, the time of acquisition is limited, and only a few transients are recorded when the LC peak crosses the flow cell; in the stop-flow mode, a significant number of transients can be acquired, consequently improving the S/N ratio. With a standard HPLC-NMR flow probe (60 μL on a 500 MHz NMR), the detection limits are about 20 μg of NP injected on-column in the on-flow mode (MW ca. 500), but they can reach the high nanogram range when HPLC-NMR is used at-line with sensitive microflow probes [14].

12.2 MODE OF OPERATION IN DIRECT AND INDIRECT LC-NMR HYPHENATION

Besides flow-probe design, the chromatographic performances and the mode of operation also strongly affect the sensitivity and quality of the spectral NMR data that can be acquired.

12.2.1 HPLC Conditions

For crude plant extract profiling, the large majority of applications from the HPLC side are performed in typical reversed-phase conditions. Gradients of acetonitrile (MeCN)–H_2O or methanol (MeOH)–H_2O with acidic or alkaline modifiers are used. For on-flow LC-NMR applications, the amount of sample loaded on the column is much greater than for typical LC-UV-DAD or LC-MS analyses, due to the inherent low sensitivity of LC-NMR. For crude plant extract analysis, the amount injected on-column can be several milligrams (see Table 12.1). In order to manage these large sample loadings, long HPLC columns (250 mm) or columns with a large inner diameter (8–10 mm) have been used for standard LC-NMR measurements [9]. Because of the poor solubility of some extracts, dissolution of the sample in deuterated dimethylsulfoxide (DMSO) is often required before such quantities can be injected on-column. As a consequence, specific chromatographic conditions are necessary for LC-NMR, in order to separate large amounts of sample with satisfactory LC resolution and maintain flow conditions compatible with the detection cell used. Another alternative is to enrich extracts in given classes of NPs on SPE columns prior to analysis. Direct combination of matrix solid-phase dispersion (MSPD) extraction with LC-NMR has also been reported [15]. Prefractionation of crude extracts on stationary phases with different selectivity than C18 might also represent an interesting alternative according to the complexity of the matrix analyzed, because the coelution of different analytes can compromise the recording of good-quality LC-NMR spectra.

If a high chromatographic resolution is required, HPLC separation without overloading can be performed. In most cases, the amount loaded will not enable the recording of satisfactory ^{1}H-NMR of minor peaks in on-flow mode. However, when using the indirect hyphenation mode, multiple collections of a given analyte can be performed and can provide efficient sample enrichment. This procedure is usually performed in LC-SPE-NMR [16].

For the analysis of very low abundance NPs in complex extracts, semipreparative HPLC fractionation of high sample amounts can be performed prior to LC-NMR analysis. Separation of the resulting enriched fractions under carefully optimized isocratic conditions provides high-resolution microfractionation (see Figure 12.10). This type of approach has been successfully applied for at-line LC-NMR measurement with sensitive microflow NMR detection methods, such as CapNMR [17].

12.2.2 On-Flow LC-NMR

The simplest way to acquire NMR data from LC peaks is to directly couple HPLC to the NMR spectrometer in what is called the “on-flow mode.” In this mode of operation, the LC-NMR spectra

TABLE 12.1
On-Flow LC-NMR Applications for NP Identification

Products	Origin	Remarks	Experiment	Instrumentation	Ref.
Azadirachtin	Photoisomerization mixture	ODS 2 (250 × 4.6 mm ID, 5 µm), inj. N.A., 1 mL/min	^{1}H	500 MHz, 60-µL flow cell	[60]
Sesquiterpene lactones	*Zaluzania grayana* and *Scalesia* spp.	ODS 2 (250 × 4.6 mm ID, 5 µm), inj. N.A., 1 mL/min	^{1}H	500 MHz, 60-µL flow cell	[65,66]
Secoridoids, xanthones, naphthoquinones	Gentianaceae spp.	C18 (150 × 3.9 mm ID, 5 µm), 1-mg inj., 1 mL/min	^{1}H, stop-flow WET-COSY	500 MHz, 60-µL flow cell	[67]
Naphthylisoquinoline alkaloids	*Ancistrocladus guineensis*	C18 (250 × 4.6 mm ID, 5 µm), 5-mg inj., 0.8 mL/min	^{1}H, stop-flow ROESY	600 MHz, 120-µL flow cell	[68]
Lignans	*Orophea enneandra*	C18 (150 × 3.9 mm ID, 5 µm), 2-mg inj., 1 mL/min	^{1}H, stop-flow COSY, NOESY, HSQC, HMBC	500 MHz, 60-µL flow cell	[19]
Prenylated flavanones	*Monotes engleri*	C18 (150 × 3.9 mm ID, 5 µm), 2-mg inj., 1 mL/min	^{1}H	500 MHz, 60-µL flow cell	[22]
2-phenylethylamine, trehalose, succinate, acetate, uridine, aristeromycin	*Streptomyces citricolor*	N.A.	^{1}H, in mixture 2-D NMR	600 MHz, 120-µL flow cell	[69]
Glycosphingolipid	Pig lungs	Normal phase (250 × 4.6 mm ID, 5 µm), 3.5-mg inj., 0.5 mL/min	^{1}H	500 MHz, 160-µL flow cell	[70]
Pyrrolizidine alkaloids	*Senecio* spp.	C18 (150 × 3.9 mm ID, 5 µm), 2-mg inj., 1 mL/min	^{1}H, stop-flow WET-COSY	500 MHz, 60-µL flow cell	[24]
Saponins	*Bacopa monniera*	C18 (125 × 5 mm ID, 5 µm), 1-mg inj., 1.0 mL/min	^{1}H, stop-flow WET-TOCSY, WET-COSY	500 MHz, 60-µL flow cell	[71]
Tocotrienol	Palm-oil extract	C30 (250 × 4.6 mm ID, 5 µm), 1-mg inj., 0.05 mL/min	^{1}H, stop-flow COSY	400 MHz, 200-µL flow cell	[72]
Ecdysteroids	*Silene otites*	C18 (150 × 3.9 mm ID, 5 µm), inj. N.A., 0.7 mL/min	^{1}H	500 MHz, 120-µL flow cell	[73]
Saponins	*Asterias rubens* (starfish), enriched fraction	ODS-L80 (250 × 4.6 mm ID, 5 µm), 1-mg inj., 0.05 mL/min	^{1}H, stop-flow WET-TOCSY	600 MHz, 120-µL flow cell	[15,74,75]
Quinone methide diterpenes	*Bobgunnia madagascariensis*	C18 (250 × 4.6 mm ID, 5 µm), 300-µg inj., 1.0 mL/min	^{1}H, stop-flow WET-TOCSY	500 MHz, 60-µL flow cell	[76]

(*Continued*)

TABLE 12.1 (CONTINUED)
On-Flow LC-NMR Applications for NP Identification

Products	Origin	Remarks	Experiment	Instrumentation	Ref.
Lignan glucosides	Flaxseed extract	C18 (250 × 4.6 mm ID, 5 µm), inj. N.A., 1.0 mL/min	^{1}H	600 MHz, 120-µL flow cell	[77]
Cardenolides	*Isoplexis* spp.	ODS-5ST (250 × 4 mm ID, 5 µm), 1-mg inj., 0.8 mL/min	^{1}H, stop-flow WET-TOCSY, WET-COSY, WET-NOESY	500 MHz, 60-µL flow cell	[78]
Prenylated isoflavones and isoflavanones	*Erythrina vogelii*	C18 (100 × 8 mm ID, 10 µm), 11-mg inj., 0.1 mL/min	^{1}H	500 MHz, 60-µL flow cell	[61]
Carbohydrates	Beer	ION-300 (300 × 7.8 mm ID, 5 µm), 100-µL inj., 0.3 mL/min	^{1}H	500 MHz, 120-µL flow cell	[79]
Furanocoumarins and quinolinealkaloids	*Stauranthus perforatus*	ODS-5ST (250 × 4 mm ID, 5 µm), 1-mg inj., 0.3 mL/min	^{1}H	500 MHz, 60-µL flow cell	[80]
Phloroglucinols	Degradation mixture	XDB phenyl (2* 250 × 4.6 mm ID, 5 µm), 6-mg inj., 0.3 mL/min	^{1}H, at line in mixture 2-D NMR	500 MHz, 60-µL flow cell	[63]
Tropane alkaloids	*Erythroxylum vacciniifolium*	C18 (125 × 8 mm ID, 10 µm), 3-mg inj., 1.2 mL/min	^{1}H, at-line 2-D NMR on main product	500 MHz, 60-µL flow cell	[57]
Labdane diterpenes and flavonoids	*Potamogeton* spp.	C18 (100 × 8 mm ID, 10 µm), 11-mg inj., 0.9 mL/min	^{1}H	500 MHz, 60-µL flow cell	[62]
Flavonoids, granaticinic acid derivatives	*Streptomyces violaceoruber* Tü 22	Phenylhexyl (150 × 4.6 mm ID, 3 µm), 500-µL inj., 0.03 mL/min	^{1}H	600 MHz, 120-µL flow cell	[81]
Phenolics	*Blumea gariepina*	C18 (100 × 8 mm ID, 10 µm), 10-mg inj., 1.0 mL/min	^{1}H	500 MHz, 60-µL flow cell	[82]
Phenolics	*Mangifera indica* (mango juice)	N.A.	^{1}H	500 MHz, 120-µL flow cell	[83]
Halogenated monoterpenes	*Plocamium mertensii* (marine alga)	C18 (250 × 4.6 mm ID, 5 µm), 1-mg inj., 1.0 mL/min	^{1}H, stop-flow, at-line COSY, HSQC	500 MHz, 60-µL flow cell	[84]

N.A., Not available.

Note: WET: Water suppression Enhanced through T1 effects; COSY: COrrelation SpectrocopY; ROESY: Rotating frame Overhauser Effect SpectroscopY; TOCSY: TOtal Correlation SpectrocopY; NOESY: Nuclear Overhauser Enhanced SpectroscopY; HSQC: Heteronuclear Single Quantum Coherence; HMBC: Heteronuclear Multiple Bond Coherence.

are continuously acquired during the HPLC separation and are stored as a set of scans in discrete increments; there is no need for complex automation to control the HPLC setup (Figure 12.1a).

In this case, the HPLC solvents must be adapted for direct NMR detection. H_2O is replaced by deuterated water (D_2O), while the organic modifier remains an HPLC-grade nondeuterated solvent (typically MeOH or MeCN), due to cost-related issues.

The use of nondeuterated solvent represents a challenging issue for NMR detection, since the signals related to these solvents will be several orders of magnitude more intense than those of the analytes of interest. Furthermore, the continuous flow of sample in the detector coil complicates solvent suppression. These problems have been overcome by the development of fast, reliable, and powerful solvent-suppression techniques, such as presaturation (Nuclear overhauser enhanced spectroscopy [NOESY] presaturation), soft pulse multiple irradiation, or WET (water suppression enhanced through T1 effects). The WET sequence, in particular, consists of a combination of pulsed field gradients, shaped radio frequence (rf) pulses, and shifted laminar pulses that can be used with ^{13}C decoupling for an efficient removal of the ^{13}C satellites within the solvent [18]. As shown in Figure 12.2, this processing sequence provides very efficient removal of solvent signals.

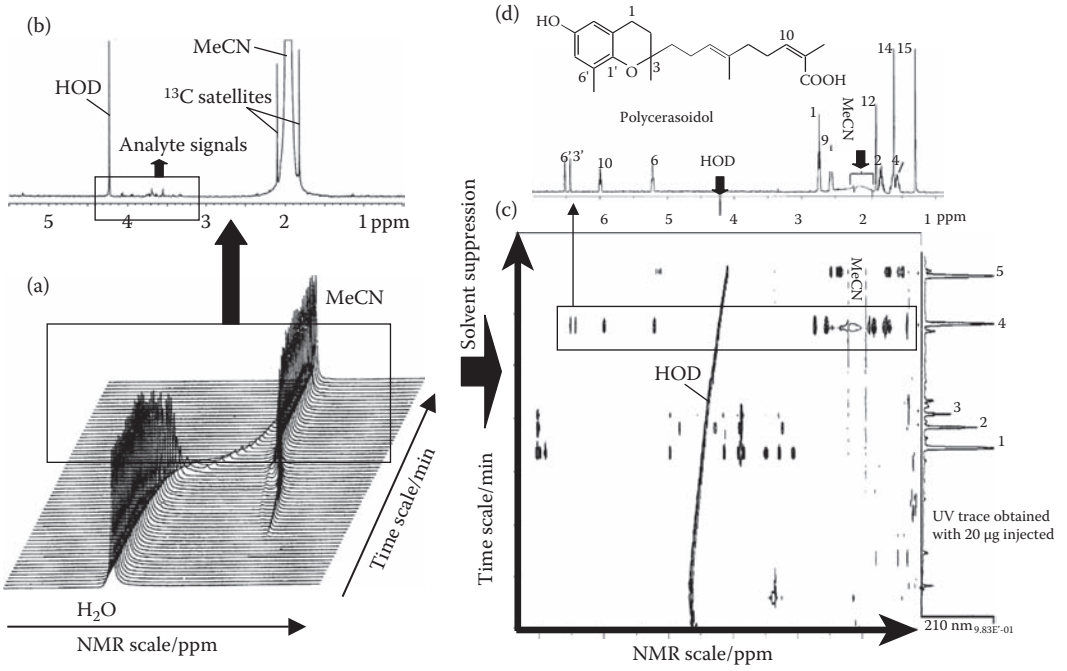

FIGURE 12.2 Principle of on-flow liquid chromatography–nuclear magnetic resonance (LC-NMR) measurements with the analysis of the enriched fraction of *Orophea enneandra* (Annonaceae). (a) Typical on-flow LC-NMR experiment without solvent suppression, which highlights the shift of the solvent signal during gradient elution. (b) Spectrum recorded in acetonitrile–deuterated water (MeCN–D_2O) without solvent suppression; the peaks of the analytes are hardly visible. (c) On-flow LC-^{1}H-NMR contour plot of the *O. enneandra* extract (2 mg injected) obtained after solvent suppression (WET sequence). All analyte signals appeared as dots. The signal of residual water signal (HOD) is negative and continually shifted during the LC gradient. HPLC conditions: Column: Nova-Pak C-18 (150 × 3.9 mm ID, 4 µm); MeCN–D_2O gradient (20:80 to 95:5 in 50 min); 0.05% trifluoroacetic acid (TFA); 1 mL/min. LC-NMR conditions: 24 scans/increment, flow cell (60 µL, 3 mm ID), 500 MHz. The UV on the side has been recorded with only 20 µg of extract injected. (d) On-flow LC-^{1}H-NMR spectrum of one of the main constituents (polycerasoidol) with regions where solvent suppression occurred highlighted. (Adapted from Bayer, E., Albert, K., Nieder, M., Grom, E., Wolff, G., and Rindlisbacher, M., *Anal. Chem*., 54, 1747–1750, 1982; Cavin, A., Potterat, O., Wolfender, J.l., Hostettmann, K., and Dyatmyko, W., *J. Nat. Prod*., 61(12), 1497–1501, 1998. With permission from the American Chemical Society.)

The problem of solvent suppression is even worse in the case of typical LC reversed-phase operating conditions, where more than one protonated solvent is used and where the resonances change frequencies during the analysis in the gradient mode. The NMR shifts encountered during a typical MeCN–H_2O gradient are illustrated in Figure 12.2a [4]. To solve this issue, the NMR maintains the main solvent signal at a fixed frequency, while the other signals are shifted. This effect of the gradient can be observed via the shift of the residual water signal (HOD) line during elution (Figure 12.2f). Satisfactory solvent-suppression efficiency can be maintained by this means throughout the whole extract separation. However, since solvent composition is always changing in on-flow LC-NMR, another problem is that the chemical shifts recorded will differ from those reported in standard deuterated NMR solvents. This can be a problem if precise comparisons with literature data have to be performed. This was the case, for example, in the analysis of various lignans from *Orophea enneandra* [19], where the chemical shifts of the aromatic protons of these compounds were significantly different, depending on whether the spectrum was recorded in on-flow conditions (MeCN–D_2O) or in standard conditions on the pure constituents (deuterated chloroform or methanol [$CDCl_3$ or CD_3OD]) [19]. Furthermore, these differences hinder the creation of LC-NMR databases for the automatic assigning of known constituents. Efforts to create an NMR database for NPs identification are ongoing, and the efficiency of such an approach has been demonstrated for the automated identification of flavonoids [20]. A typical on-flow spectrum of a tocopherol derivative (polycerasoidol, Figure 12.2d) recorded in the same extract shows the domain where solvent suppression of both HOD and MeCN signals occurs.

Another limitation of on-flow LC-NMR is its low sensitivity, which is also dependent on the number of transients per increment that can be acquired (typically 4 to 32) for a standard LC-NMR flow rate (1 mL/min). This number of transients can be increased if the flow rate is decreased, which will increase the residence time of the analytes in the flow cell. For relatively low flow rates (0.1 mL/min), the number of transients per increment can increase to 256 or 512 [21].
According to the analysis, a compromise between the number of scans per increment and the LC resolution must be made to ensure both a satisfactory sensitivity and a reasonable separation of the signals of closely eluting LC peaks.

Practical on-flow LC-NMR is restricted to the recording of the NMR spectra of the main constituents of a crude extract, and this often necessitates "overloaded" LC conditions. Typically, 1–5 mg of crude plant extract is injected on-column. In the on-flow analysis presented in Figure 12.2c, 2 mg of dichloromethane extract was injected. This mode of operation can be used to rapidly obtain structural information on the main constituents of an extract and is particularly useful for the study of unstable NPs.

The detection limits in the on-flow mode with a standard LC-NMR flow probe (60 µL on a 500 MHz) NMR is about 20 µg on-column, while a ^{1}H-NMR spectrum of satisfactory quality is obtained with about 150 µg. An example of a typical on-flow spectrum obtained for the secoiridoid sweroside (150 µg) is shown in Figure 12.3a and is compared to that obtained in the stop-flow mode.

12.2.3 Stop-Flow and Loop Storage

One way to improve the detection limits of LC-NMR in direct hyphenation is to work in the stop-flow mode. Operation in stop-flow mode requires that the retention times of the analytes of interest are known, or that a sensitive method of detection such as LC-UV or LC-MS is used prior to LC-NMR to trigger the detection. In practice, one of these detectors is connected on-line before the NMR instrument, and the signal of the analyte of interest passing through this detector is used to trigger a valve that will stop the LC flow once the peak reaches the NMR cell, after a calibrated delay (see experimental arrangement, Figure 12.1b). The stop-flow mode provides the possibility of acquiring a significant number of transients on a given LC peak, and satisfactory ^{1}H-NMR spectra of compounds present in the low microgram range can be obtained. The solvent suppression is also of a better quality in the stop-flow mode than in the on-flow mode. During a given separation,

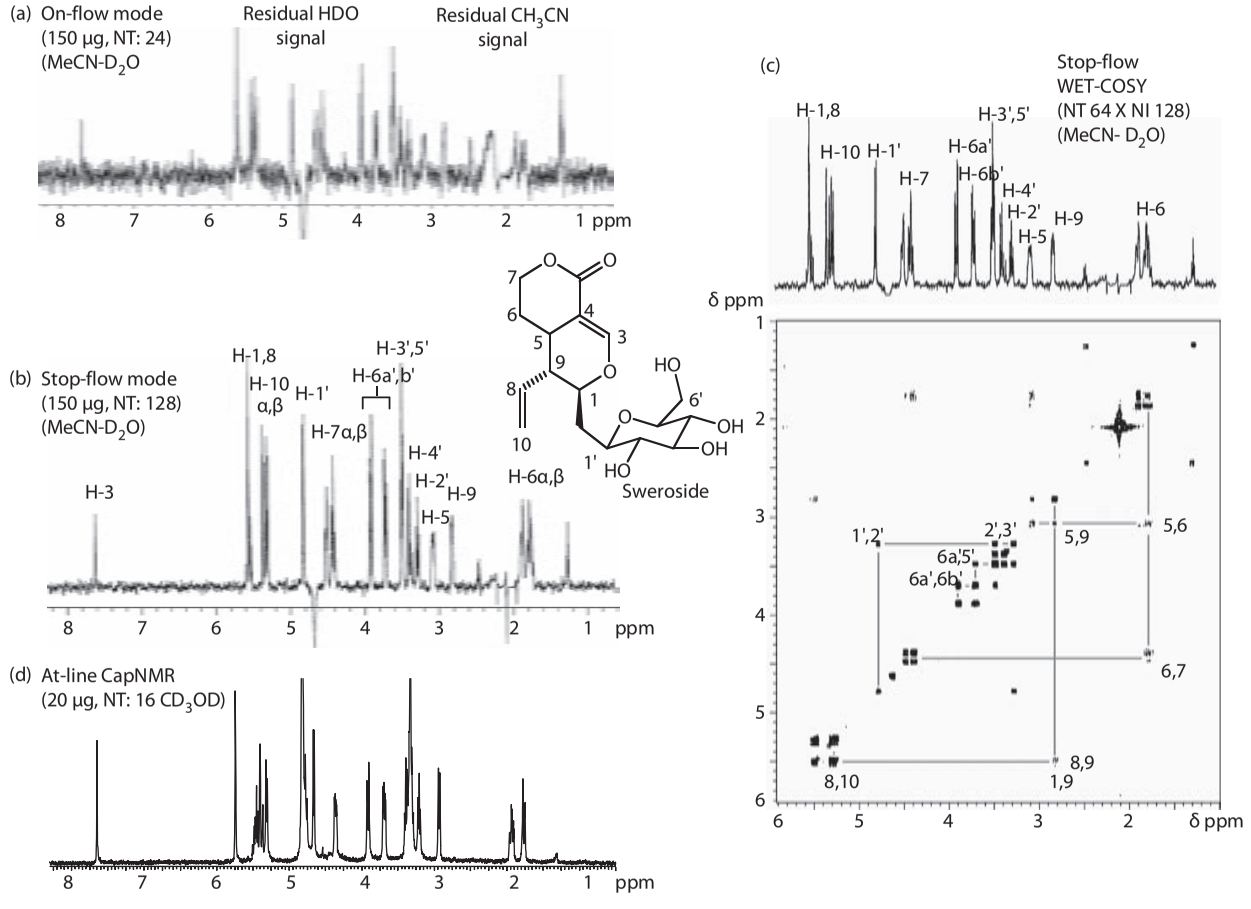

FIGURE 12.3 Comparison of the (a) on-flow and (b) stop-flow liquid chromatography–^{1}H-nuclear magnetic resonance (LC-^{1}H-NMR) of sweroside (150 µg in MeCN–D_2O) with direct acquisition of a WET-COSY ^{1}H-^{1}H stop-flow spectrum (c). LC-NMR: 60 µL flow cell (3 mm ID); 500 MHz. (d) Capillary NMR (CapNMR) spectrum recorded for a similar compound, swertiamarin (20 µg), recorded in 5 µL of CD_3OD. (WET: water suppression enhanced through T1 effects; COSY: COrrelation SpectrocopY; D_2O: deuterated water; CD_3OD: deuterated methanol; MeCN: acetonitrile.) (Adapted from Wolfender, J.l., Rodriguez, S., Hostettmann, K., and Hiller, W., *Phytochem. Anal*., 8(3), 97–104, 1997. With permission from Wiley.)

several stop-flow experiments can be performed. In gradient elution, the interruption of the flow rate usually does not considerably alter the chromatographic separation; however, peak broadening can be observed if the separation is performed in the isocratic mode. 2D correlation experiments such as correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), and NOESY can be acquired in stop-flow, but since no analyte preconcentration is possible in this direct hyphenation mode, sensitive ^{1}H-^{13}C correlation spectra are difficult to acquire in practice [22].

Measurement of stop-flow experiments can be automated using what is called the "loop storage" mode. In this case, a loop collector, triggered by a sensitive LC detector (LC-UV or LC-MS), will automatically collect the peaks of interest without stopping the LC flow. Off-line postchromatographic analysis of the content of the loops is then automatically performed in LC-NMR (see experimental arrangement, Figure 12.1c) [23]. This mode of operation represents a more practical way to perform stop-flow analyses, but it requires rather complex automation.

An example of the improvement in S/N ratio obtained between the on-flow and stop-flow modes is shown in Figure 12.3b for the secoiridoid sweroside. Here, about 150 μg of the compound was injected. As shown, in this mode, it was possible to obtain ^{1}H-^{1}H 2D correlation (COSY) directly on the corresponding LC peak (Figure 12.3c). It is, however, important to note that the quality of the spectra obtained in the stop-flow mode is lower than that obtained in the indirect hyphenation of NMR (see the following). For comparison purposes, the ^{1}H-NMR spectrum of a similar secoiridoid, swertiamarin, obtained with only 20 μg with CapNMR, is displayed in Figure 12.3d.

As already discussed, direct hyphenation of NMR with HPLC presents some advantages, such as the ability to obtain NMR information directly on a given NP without further handling of the corresponding LC peak. However, this mode of operation requires compromises that alter the quality of the data in comparison with conventional NMR experiments. Indeed, first, the elution volumes of the peaks are often bigger than the volume of the flow cell, which decreases sensitivity. Second, because of the need for solvent suppression, the analyte signals of interest, which reside under the solvent peak, are also suppressed, and this restricts the observable NMR range [19]. The missing structural information can be obtained by performing a complementary analysis in a different solvent system, such as MeOH–D_2O, and this has been reported for the analysis of various pyrrolizidine alkaloids in *Senecio* species [24]. The suppression of the analytes' signals can be solved by performing the separation in fully deuterated solvents (D_2O–CD_3CN), but the cost of such analysis can be high if typical LC conditions (4-mm ID columns, 1 mL/min flow rate) are used. However, this approach can be applied to microcolumns, where the solvent consumption is limited [25]. Third, the chemical shifts recorded in a typical reversed-phase solvent (MeCN–D_2O or MeOH–D_2O) will differ from those reported in standard deuterated NMR solvents. This can be a problem if precise comparisons with literature data or searches in databases are to be performed.

In order to cope with the limitations related to the direct hyphenation of NMR, analytical strategies based on the indirect hyphenation of this technique have been devised [9,16,26,27]. These at-line strategies represent a new trend in NMR hyphenation that is mainly directed toward increased detection sensitivity and higher quality of data; however, they require more sample handling of the LC peaks of interest. In Figure 12.4, the possibilities and limitations related to this passage from on-line LC-NMR in hyphenated systems to at-line automated methods are summarized.

12.2.4 LC-SPE-NMR

One of the major breakthroughs in NMR hyphenation has been the introduction of the LC-SPE-NMR technique, which enables an efficient preconcentration of the sample prior to the LC-NMR spectra. The principle of the method was initially described about a decade ago [28].

In LC-SPE-NMR, the separated peaks are diluted postcolumn with water and trapped automatically on a preequilibrated on-line SPE cartridge [23]. After a drying step with nitrogen to remove all of the solvents used for chromatographic separation, the analytes are transferred to the NMR flow cell with a deuterated solvent of choice that has sufficient elution power (see experimental

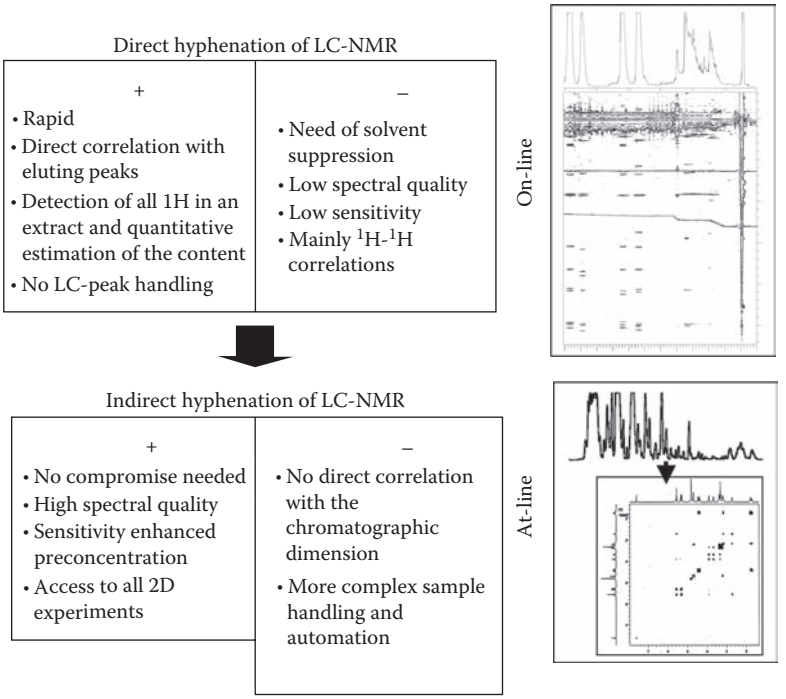

FIGURE 12.4 Advantages (+) and limitations (–) of direct and indirect hyphenation of nuclear magnetic resonance (NMR) with HPLC.

arrangement, Figure 12.1d) [26]. The main advantage of the method is that the sample, which might elute as a relatively broad peak from the column, is trapped and concentrated to an elution volume that is close to the flow-cell volume of the LC-NMR probe. In this way, all NMR-active nuclei will be concentrated in the volume of the flow cell, and thus, the sensitivity will be increased. In direct hyphenation, it is indeed known that the concentration in later LC eluting peaks is reduced by a factor of at least 3, due to the diffusion of the LC chromatographic process, and NMR detection of these dilute solutions would require a very high detection volume (in the milliliter range) [2].

Another important advantage of LC-SPE-NMR is that HPLC separation is cost effective, since it can be carried out with normal protonated solvents. Additionally, the appropriate deuterated solvents can be used for NMR detection without the need for solvent suppression. LC-SPE-NMR also allows multiple trappings of the same analyte from repeated LC injections. This mode of operation enables the circumvention of problems, such as those with sample solubility and HPLC column loadability, which are often encountered in standard LC-NMR hyphenation, especially during the detection of minor metabolites.

Successful postcolumn HPLC focusing of the LC peaks, however, requires a good trapping efficiency of the analyte of interest and careful optimization of these conditions. In this respect, the physicochemical properties of the analytes must be considered in selecting the best SPE trapping material and the nature and the amount of the added make-up flow [29]. For most of the applications reported herein, the stationary SPE phases consisted of a divinylbenzene-type polymer or C18 material, and the trapping eluent was H_2O (1–2 mL/min) (Table 12.3). The transfer of the analyte as a sharp elution band from the SPE is also strongly dependent on the nature of the deuterated solvent used.

The whole operation can be automated in integrated state-of-the-art systems, which is extremely useful if multiple trapping is needed or if the HPLC chromatographic profiling of crude extra requires separations that might exceed one hour [30]. The optimum separation strategy might

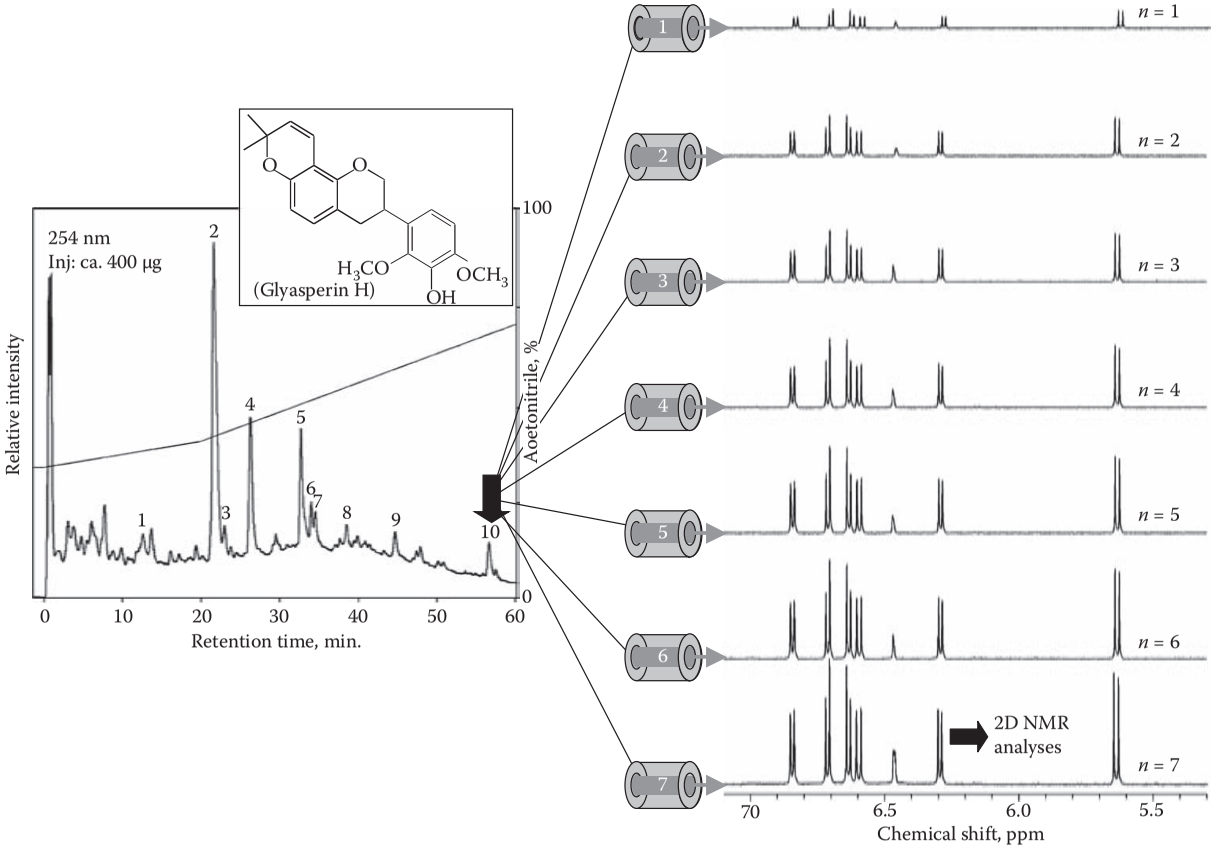

FIGURE 12.5 Multiple solid-phase extraction (SPE) trapping ($n = 1$–7) of glyasperin H, a *Smirnovia iranica* secondary metabolite, resulted in a linear increase of the signal-to-noise ratio (S/N). This minor metabolites was isolated directly from the crude extract (see inset), after sixfold trapping ($n = 6$). Two-dimensonal nuclear magnetic resonance (2D NMR) spectra were recorded. HPLC conditions: Inj. 10 mg; Col. Phenomenex Luna C18 (150 × 4.6 mm ID, 5 µm); MeCN–H_2O gradient, 0.5 mL/min. SPE-NMR trapping on SPE C18 HD (10 × 2 mm ID), elution CD_3CN, flow cell (60 µL, 3 mm ID), 600 MHz. (MeCN: acetonitrile; CD_3CN: deuterated acetonitrile.) (Adapted from Lambert, M., Staerk, D., Hansen, S.H., Sairafianpour, M., and Jaroszewski, J.W., *J. Nat. Prod*., 68(10), 1500–1509, 2005. With permission from the American Chemical Society.)

change according to the polarity of the analyte, and the use of either semipreparative or analytical columns with or without multiple trapping has been investigated [31].

An example of the effect of multiple trapping on sensitivity is illustrated using the analysis of isoflavonoids from *Smirnovia iranica* in Figure 12.5. The ^{1}H-NMR spectrum of glyasperin H (peak 10), a representative compound present in quite small amounts, is displayed. As is shown, up to seven repeated trappings of peak 10 resulted in a linear increase of the S/N ratio in the NMR spectra [32].

Compared to stop-flow LC-NMR, the increased sensitivity provided by analyte-focusing multiple trapping and the advantage of full deuterated solvent operation give the ability to perform the full set of 2D correlation experiments on the analytes, including heteronuclear multiple-bond correlation (HMBC), in a reasonable analysis time. An example of the type of 2D NMR spectra that can be obtained is illustrated for the sesquiterpene lactone thapsigargicin in Figure 12.9 [33].

A typical LC-SPE-NMR, which would need a full set of 2D spectra, will require about 120 μg in a 60 μL cell on a 600 MHz equipped with a room-temperature probe head. For a secondary metabolite (MW < 700 Da), a series of high-quality homonuclear and heteronuclear 2D spectra (e.g., a combination of DQF-COSY [double-quantum-filtered COSY], TOCSY, HSQC [heteronuclear single quantum correlation], and HMBC) were recorded overnight. Single ^{1}H-NMR spectra can be obtained with about 10 nmol of substances within a reasonable timeframe [34].

The sensitivity of the flow cell can be enhanced by using a cryogenated probe. The use of new cryoflow probes also considerably enhances the sensitivity by a factor of roughly 3–4 compared to noncryogenated systems [35]. For LC-NMR, cryofit inserts for the adaptation of 30 μL probes in a 3 mm ID inverse cryoprobe have been successfully used for the analysis of very limited amounts of plant material [36].

12.2.5 Microflow Capillary NMR (CapNMR)

As already mentioned, one of the last improvements in flow-cell design has been the introduction of reduced-diameter solenoid NMR coils (CapNMR), which represents a particularly attractive approach to enhance NMR sensitivity for mass-limited sample uses [12]. Similar to LC-SPE-NMR, CapNMR represents another alternative for the indirect hyphenation of NMR with HPLC. For analyzing extracts, the same microfractionation procedure as that described for LC-SPE-NMR can be used; however, in this case, the LC peaks are not trapped on SPE cartridges but simply collected into vials or in 96-well plates. As with LC-SPE-NMR, simple or multiple collection of a given analyte is possible, but in this case, no optimization of SPE trapping conditions is required, and all peaks are dried from the 96-well plates, usually by speedvac evaporation. Each sample can be manually dissolved in a volume slightly exceeding the volume of the flow cell (6.5 μL for a 5-μL flow-cell volume), filtered, injected into the probe, and parked in the center of the microcoil by pushing it with an adjusted push volume (typically 8–10 μL) [9,37] .

An example of the type of spectra that can be recorded by CapNMR after microfractionation is illustrated in Figure 12.6b. In this case a comparison between an on-flow ^{1}H-NMR spectrum (Figure 12.6a) recorded for a constitutive flavonoid triglycoside of *Arabidopsis thaliana* obtained directly upon injection of the crude extract and the analysis of the same product that has been microfractionated, dried, and injected in CapNMR (Figure 12.6b) is presented. As is shown, a significant improvement in spectral quality and sensitivity is obtained, and no solvent suppression was necessary.

As in the case of LC-SPE-NMR, all operations for CapNMR can be fully automated [38], and a very efficient transfer can be obtained. In this respect, a thoroughly optimized automated droplet microfluidic NMR loading method (microdroplet NMR) has been recently reported. It enables performance of NMR offline from LC-UV-MS, accommodates the disparity in sample mass and time

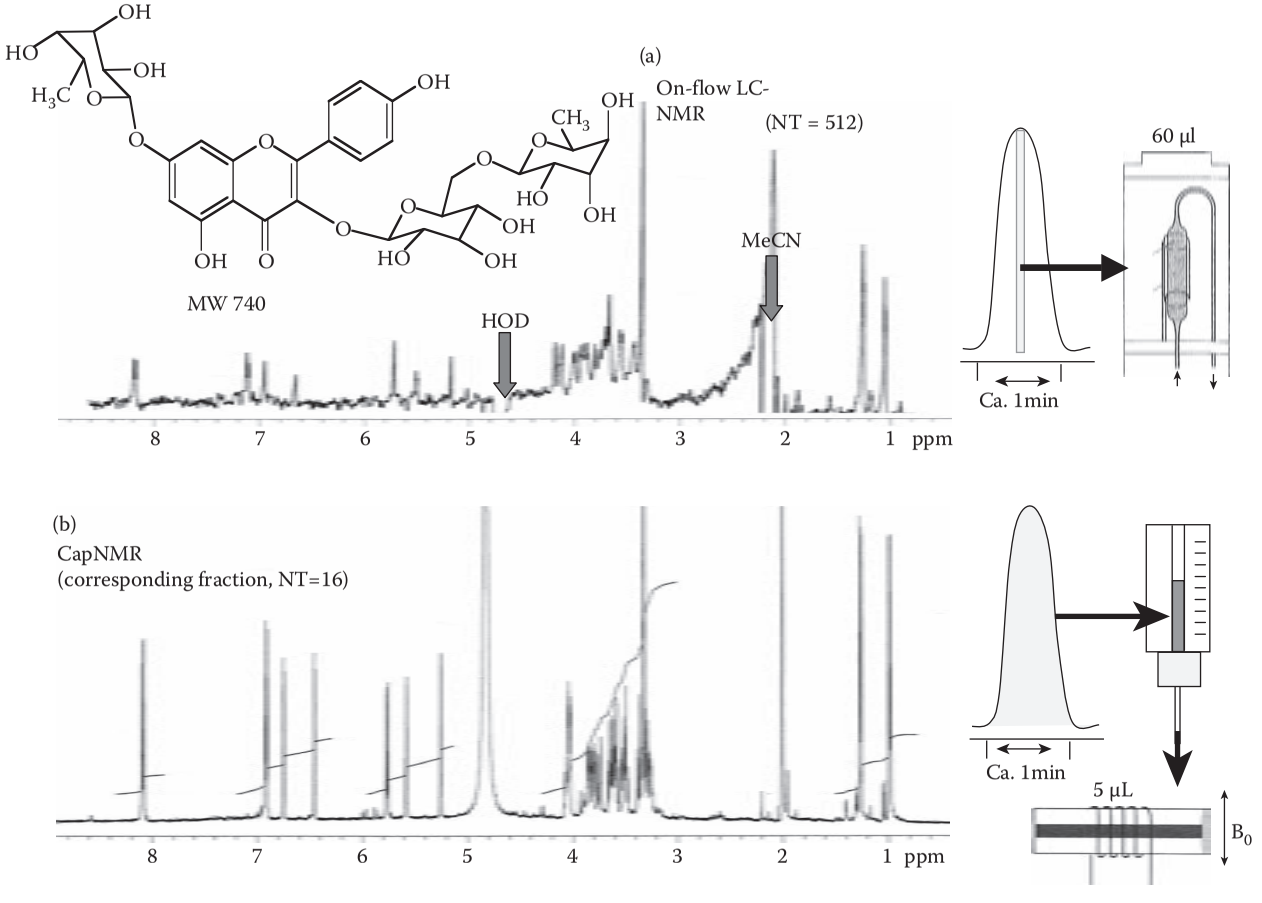

FIGURE 12.6 Comparison between on-flow liquid chromatography–nuclear magnetic resonance (LC-NMR) and capillary nuclear magnetic resonance (CapNMR) spectra of a flavonoid triglycoside from the crude extract of *Arabidopsis thaliana* (ca. 20 µg). (a) On-flow LC-NMR in MeCN–D_2O (NT = 512), 60-µL observe volume flow cell (Varian, Palo Alto, Calif., USA); 500 MHz. (b) CapNMR spectra in CD_3OD (NT = 16); the sample was dissolved in 6.5 µL of CD_3OD and parked in the probe with a push volume of 5 µL, 1.5 µL observe volume flow cell (Protasis/MRM Corp., Savoy, Il.); 500 MHz. Insets: Schematic showing the increase in concentration from on-flow to at-line CapNMR on a typical LC peak. (MeCN: acetonitrile; D_2O: deuterated water; CD_3OD: deuterated methanol.)

requirements between MS and NMR, and allows NMR spectra to be requested retrospectively, after review of the LC-MS data [39].

With such an approach, interpretable 1D NMR spectra were obtained from analytes at the 200 ng level in 1 h well-automated NMR data acquisitions. When applied to a cyanobacterial extract showing antibacterial activity, the platform recognized several previously known metabolites, down to the 1% level, in a single 30-μg injection, and prioritized one unknown for further study [39]. With the CapNMR approach, typically, a few tenths of a microgram (depending on the magnet strength and MW) are needed to obtain a full set of 2D correlations overnight. On a 600 MHz system, on a routine basis, COSY NMR spectra were reported to be acquired with less than 10 μg in about 1 hour; 30 μg were needed for HSQC in 5 hours, and 70 μg for HMBC in about 15 hours. Direct ^{13}C-NMR data can already be obtained with 200 μg of NPs [37].

12.2.6 SPE-CapNMR

One of the limitations of CapNMR might be related to the redissolution of the dried analyte in small amounts of deuterated solvents, in which the sample might reach a high concentration (up to ca. 40 mM) for the recording of sensitive experiments, such as HMBC. In this respect, the combination of SPE-NMR, which is normally suited for use with 30–60 μL probes, has been investigated with CapNMR. Elution profiles of 10 × 1 mm ID SPE cartridges (instead of 10 × 2 mm ID cartridges for conventional SPE-NMR) were found to be only marginally broader than those observed upon direct injection of 6-μL samples into the probe, thus rendering SPE-CapNMR practically applicable for crude plant extract profiling studies. An example of the type of data generated by this approach is discussed below and is shown in Figure 12.9, for the structure elucidation of sesquiterpene lactones and esterified phenylpropanoids [33]. The method thus combines the advantage of high loadability of normal-bore HPLC columns with the high mass sensitivity of capillary NMR probes possessing an active volume of 1.5 μL.

12.2.7 Other At-Line Sensitive Approaches Using Nonflowing Cells

Since the trend in LC-NMR hyphenation is to perform NMR measurement in the indirect hyphenation mode, the concept of LC-NMR can also be extended to other sensitive, micro-NMR methods, provided that a direct relation to the LC peaks of the analytes at the analytical scale is maintained. The transfer from HPLC peak collection is thus not necessarily made with fluidics. In this respect, microprobes using disposable 1 mm capillary sample tubes, having a sample volume of 5 μL, also represent an interesting approach to the analysis of limited analytes collected from HPLC separation [40].

12.3 COMBINATION OF LC-NMR WITH OTHER DETECTORS ("HYPERNATION")

As already shown, according to the mode of operation, LC-NMR can provide high-quality NMR data on NPs with relatively high sensitivity. However, even if NMR is arguably the most versatile analytical platform for complex-mixture analysis [23], it is seldom possible to solve the structure of a novel compound by this technique alone. Indeed, common functional groups such as carboxylic acids, phenols, and amino groups are NMR silent in many solvents because of proton-deuterium exchange. The presence of these groups, however, can be deduced from MS, which will provide molecular weight, molecular formula, and fragment information [23]. In NP chemistry, UV spectra may also bring very valuable information, especially for polyphenols, which exhibit characteristic chromophores [41]. In drug discovery platforms, MS or UV detections are also very important for triggering the specific collection of a given analyte prior to sensitive NMR measurements [17,27,30]. Furthermore, other existing hyphenated techniques such as LC-IR (IR: infrared) [42] or LC-CD (CD: circular dichroism) [43] may also bring valuable complementary information.

12.3.1 Complementary LC-DAD-UV and LC-MS Analyses from Independent Analyses

In LC-NMR, LC-DAD-MS is often performed as a separate analysis because of the constraints of each detection method. LC-DAD-MS is used as a first dereplication step for the chemical profiling of crude plant extracts, and compounds are tentatively identified based on molecular weight and fragment information, followed by a search of NP libraries and by matching to UV in-house spectral libraries [44]. LC-NMR is mainly used as a second step for a more detailed structural investigation of compounds presenting original structural features or displaying interesting activities after LC bioassays [45].

The use of LC-MS is widespread for crude extract profiling [8], and it is a key technique for the on-line identification of NPs. This technique gives the possibility of generating either nominal mass molecular ions or accurate mass measurements for the determination of empirical formulas [46]. Furthermore, the use of tandem or hybrid MS instruments [47] provides in-depth structural information through fragmentation of the molecular species by collision-induced dissociation (CID) reactions. Several reviews describe aspects of on-line NP identification for screening and dereplication [8,14,48–51].

DAD-UV spectra, on the other hand, provide initial information regarding the type of UV-active NPs present in extracts, since some of the secondary metabolites of extracts exhibit characteristic chromophores. For polyphenol analysis, for example, the postcolumn addition of different UV-shift reagents [52], with multiple injection of a given plant extract, can be performed on-line. The analysis of the set of UV-shifted spectra for each separated polyphenol provides valuable additional on-line information regarding the position of hydroxyl groups [53,54].

12.3.2 Integrated Systems with Multiple Detection

It has been shown to be feasible in practice to acquire NMR, UV, IR, and MS spectra based on a single HPLC analytic run, in what has been termed a "hypernated" system (LC-DAD-FTIR-NMR-MS) (FTIR: fourier transform infrared). This approach has permitted, for example, the characterization of ecdysteroids from various silene species [55]. In these hypernated systems, the difficulties center around finding eluants that are compatible with all of the spectroscopic techniques involved, coping with large mutual differences in analyte delectability as well as in data handling [56].

The more robust hypernated systems, which also have evolved into completely automated systems, are those involving LC-NMR-MS [23]. The main problems for such platforms arise from sample overloading into the MS systems and a shift of the molecular ion species due to proton-deuterium exchange reactions. These types of problems, however, can be solved by using an efficient postcolumn splitter, which diverts a small portion of the flow into the mass spectrometer and enables proton-deuterium back exchange by dilution with an appropriate make-up flow [15]. Deuterium exchange experiments on the MS side might also provide valuable additional structural information on the number of exchangeable protons of a given molecule [57]. The great advantage of LC-NMR-MS is that MS data can be directly correlated to the NMR spectra acquired. LC-MS can also be used to precisely trigger stop-flow or loop storage LC-NMR experiments. Furthermore, the MS detection can be used to deconvolute coeluting components with a limitation for isobaric or isomeric metabolites.

The latest developments in the indirect hyphenation of LC-NMR involve automated MS-directed SPE trapping in LC-SPE-NMR-MS setups [26] or MS-directed microfractionation for further CapNMR measurements [17]. Both approaches provide a sensitive and selective detection of given metabolites and ensure a precise collection of the analytes prior to NMR measurements.

In most applications, UV, MS, MS/MS, and NMR data of given analytes are often gathered for the full or partial structure elucidation of the metabolites of interest in crude plant extracts. Applications also include circular dichroism (CD) measurements to the set of data to include chirality characterization of the NPs of interest [58,59].

12.4 APPLICATIONS OF ON-FLOW AND STOP-FLOW LC-NMR TO NP ANALYSIS

As already discussed, the limited residence time of the analyte in on-flow LC-NMR affects the spectral quality and limits the sensitivity of the technique. Thus, on-flow LC-NMR applications are mainly used to give an overview of the major metabolites present in a plant extract and their real proportion, since NMR can be considered an absolute quantification method. Often, an LC-NMR experiment is used as an introductory experiment followed by investigations in the stop-flow mode [7,9].

A seminal experiment in 1994 has demonstrated the applicability of on-flow LC-NMR to the analysis of NPs, with an application related to the study of photoisomerization of azadirachtin from the seeds of the neem tree [60]. This study was performed on a 500 MHz NMR equipped with a 60 μL flow probe, and the on-flow results enabled an unambiguous determination of the photoproducts.

Since then, many on-flow experiments have been reported (see Table 12.1). Most of the studies were performed in gradient mode, using HPLC C18 columns of large diameter (10 × 8 mm) [45] or long columns (250 × 4.6 mm) [60] with injection of a sizable amount of extract (ca. 10 mg). While most of the studies were performed with standard HPLC flow rates (Table 12.1), extracts were sometimes eluted at very low flow rates (down to 0.1 mL/min) to increase analyte residence time in the flow cell used [57,61–63]. The isocratic mode with CH_3CN–D_2O mixtures was also used to detect naphthylisoquinoline alkaloids in crude plant extracts [64].

The direct coupling of LC-NMR was found to be particularly useful for the structure elucidation of unstable NPs. HPLC-UV-MS and HPLC-NMR analysis of extracts containing compounds that could be isolated in pure form were thus successfully obtained [85,86].

This was the case, for example, for the study of cinnamic acid derivatives, which undergo light-induced *cis-trans* isomerization when tentatively isolated at the preparative scale [85]. The same type of approach was applied to the study of aucubin derivatives that undergo transacylation reactions of their cinnamoyl moiety on the rhamnose units during isolation [86].

On-flow LC-NMR was also efficient for the differentiation of closely eluting oxidized forms of hyperforin [63]. Two isomers of a "quinone methide" diterpene with a cassane skeleton, which were interconverting in solution, were also fully identified based on a similar approach [76].

On-flow LC-NMR spectra alone do not always provide a complete identification of NPs, but the method is very efficient for the detection of different variations of the core structure that occur in a plant extract. In this case, the complete structure elucidation of a representative NP can be performed by the isolation of a main compound and the different derivatives characterized based on comparison of on-flow ^{1}H-NMR spectra. This approach was used for the analysis of the alkaloid from *Erythroxylum vacciniifolium*, a Brazilian Erythroxylaceae known as "catuaba" [87]. In this case, after a complete identification of the main tropane alkaloid (catuabine D) by standard 5 mm 2D NMR experiments, comparison of the ^{1}H-NMR spectra of the different derivatives enabled their identification on-line.

On-flow studies have not been performed on plant constituents only; this technique was also found to be useful for the investigation of secondary metabolite products of microorganisms, such as those from *Streptomyces violaceoruber* TU 22, where more than 50% of the identified metabolites were new compounds [81].

An example of the integration of the on-line results that can be brought by LC-UV-MS and LC-NMR is shown by the investigation of the dichloromethane extract of *Erythrina vogelii* Hook. f. (Leguminosae) [61,88], which exhibited a strong antifungal activity in thin-layer chromatographic bioautography against the plant pathogenic fungus *Cladosporium cucumerinum* [89]. The use of this bioassay at-line was also helpful for linking the bioactivity to peaks identified on-line.

The extract of *Erythrina vogelii* was first analyzed by high-resolution LC-Q-TOF (quadrupole time-of-flight)-MS/MS, together with LC-UV-DAD with postcolumn addition of UV shift reagents.

The LC-UV trace revealed the presence of a dozen major peaks. These different constituents shared rather similar types of UV spectra, with two main absorption bands of decreasing intensity characteristic for isoflavones. From the high mass accuracy LC-MS data, the molecular formula could be assigned (precision <5 ppm) [61]. The presence of fragments due to losses of 56 and 69 Da in the LC-MS/MS spectra of most of the constituents revealed the possible presence of prenyl chains (see Figure 12.7e). From these preliminary LC-UV-MS results, it could be concluded that the CH_2Cl_2 extract of *E. vogelii* most probably consisted of a combination of various prenylated isoflavanones or isoflavones.

The on-flow LC-NMR analysis of the extract was carried out by injecting a large amount of the extract on column, and the sensitivity of detection was enhanced by conducting the separation at a low flow rate, for the accumulation of a large number of transients across the LC peaks. On the on-flow contour plot of the crude extract, the signals of more than 10 LC peaks were efficiently recorded (Figure 12.7a).

During LC-NMR analysis, an LC microfractionation of the different peaks was performed every 10 min (1 mL fractions) for the antifungal bioautography assays against *C. cucumerinum*. The postchromatographic antifungal assay revealed that the fractions associated with the LC peaks eluting at 8.4, 10.8, 11.3, 12.2, and 14.6 hours in the low-flow LC-NMR experiment display distinguishable antifungal activities (Figure 12.7).

The ^{1}H-NMR data extracted from the low-flow LC-NMR analyses confirmed that all the major constituents of the crude extract of *E. vogelii* were prenylated isoflavones and isoflavanones (Figure 12.7a). A first evaluation on the on-flow LC-NMR contour plots revealed that in all chromatographic peaks, aromatic signals between 5.9 and 6.4 ppm were indicative of an A-ring oxygenated in positions 5 and 7. This was in good agreement with chemotaxonomical data, which indicated a 5,7,4′ oxygenation for biosynthetic reasons. For the more polar constituents, methylene protons at 4.39–4.57 ppm were characteristic of an isoflavanone nucleus, while most of the peaks eluting in the second half of the chromatogram displayed aromatic singlets at ca. 8 ppm, indicative of isoflavones. The presence of two groups of flavonoids was also confirmed by the presence of two distinct types of chromophores in the UV-DAD spectra recorded. Signals of prenylated groups were detected between 3.0 and 3.3 ppm (H-1″), 5.1 and 5.3 ppm (H-2″), and 1.5 and 1.6 ppm (2 CH_3) for most of the compounds. As an example, the on-line data obtained for the isoflavone that presented the strongest zone of inhibition in the postchromatographic antifungal bioautography assay are presented in Figure 12.7. Its molecular formula of $C_{20}H_{19}O_5$ was deduced from LC-APCI (atmospheric pressure chemical ionization)-TOF-MS (*m/z* 339.1208 $[M + H]^+$; Figure 12.7d), while losses of C_4H_7 or C_5H_9 were characteristic for a prenyl moiety in the MS/MS spectrum of the $[M + H]^+$ ion (Figure 12.7f). In the on-flow LC-^{1}H-NMR spectrum, a singlet at δ 7.9 was assigned to the H-2 proton, characteristic of an isoflavone nucleus. The substitution pattern could be deduced from the aromatic signals (δ 6.0–7.5; Figure 12.7c). The positions of the three hydroxyl groups and the prenyl unit were deduced from the complementary UV-shifted spectra and the MS/MS experiments. The shifted UV spectra (Figure 12.7f) were obtained by LC-UV-DAD analyses of the extract with postcolumn addition of UV shift reagents [90] and analyzed according to well-established rules for pure flavonoids [52]. For this isoflavone a 5,7-dihydroxylation of the A-ring was deduced from spectra recorded with $AlCl_3$ and NaOAc (Figure 12.7f). The obtained CID MS/MS spectra confirmed the presence of two hydroxyl groups on the A-ring (RDA cleavage *m/z* 153.0450 $[A1]^+$; Figure 12.7e) [91]. All of these data suggested that it was isowighteone, a known prenylated isoflavone that was dereplicated by this means [61].

De novo structure determination based on complementary sets of on-line data obtained was performed for each LC peak of interest, and eight polyphenols were thus partially or totally identified on-line. Some of these compounds were found to be new NPs, and their targeted isolation was undertaken [88].

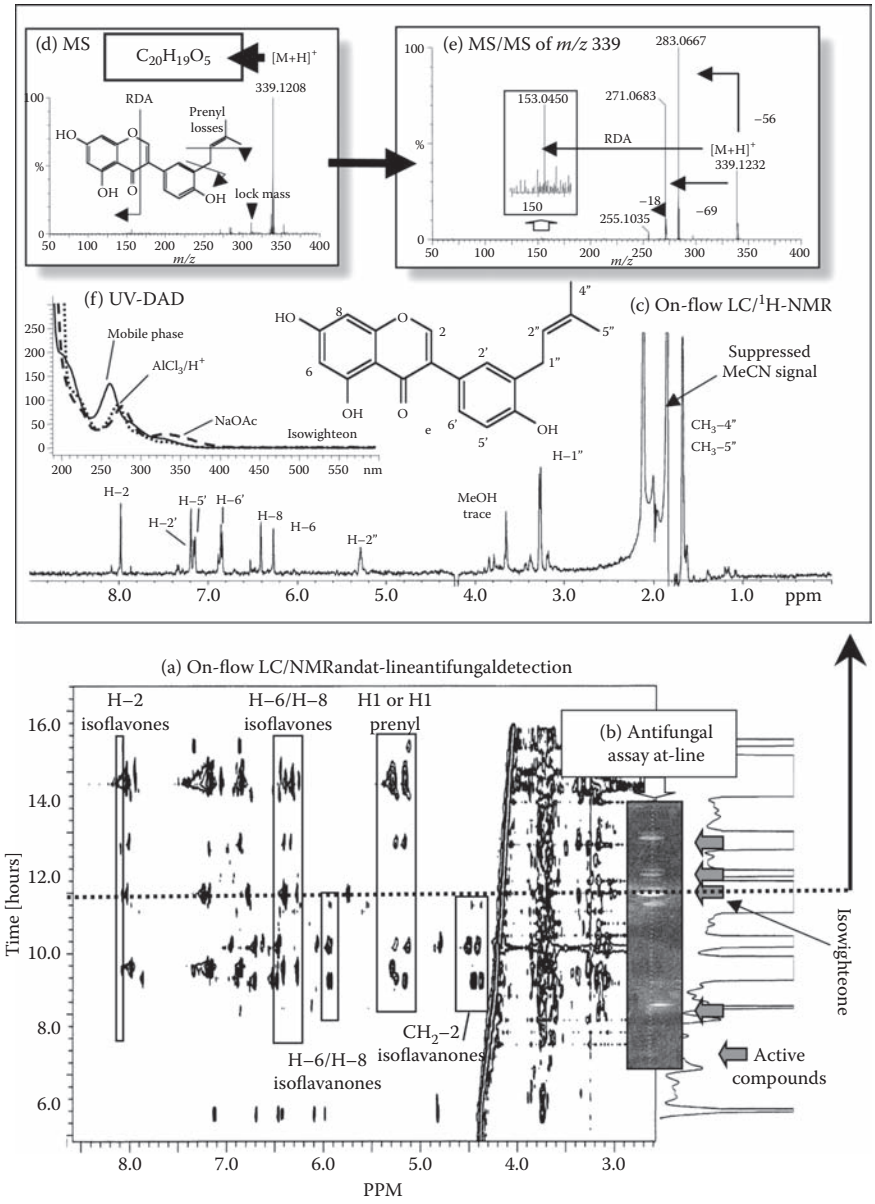

FIGURE 12.7 (a) On-flow liquid chromatography–nuclear magnetic resonance (LC-NMR) contour plot of the crude CH_2Cl_2 extract of *Erythrina vogelii* (Leguminosae) showing the ^{1}H-NMR resonances of all the main constituents. Several characteristic regions for the resonances of isoflavanones and isoflavones are highlighted. (b) Inset with the result of the antifungal assay performed on the LC microfraction against *Cladosporium cucumerinum*. (c) On-flow LC-^{1}H-NMR spectrum of the main antifungal agent isowighteone **1** (NT 265). (d) LC–quadrupole time-of-flight mass spectrometry (Q-TOF-MS) spectrum of **1** (calc mass 333.1232 for $C_{20}H_{19}O_5$, error -2.5 mDa). (e) LC-Q-TOF-MS/MS of *m/z* 339 showing typical loss for prenyl moieties and an RDA fragment characteristic for an A-ring with two hydroxyl groups. (f) UV-photodiode-array (DAD) spectra of 1 after postcolumn derivatization with $AlCl_3$ and NaOAc; shifts are characteristic for a 5,7-dihydroxylation of the A-ring. HPLC conditions: Inj. 10 mg; Col. μ-Bondapak C-18 (100 × 8 mm ID, 10 μm); MeCN–D_2O gradient (5:95 to 100:0; 19 hours).; 0.05% trifluoroacetic acid (TFA); 0.1 mL/min. LC-NMR conditions: 256 scans/increment, flow cell (60 μL, 3 mm ID), 500 MHz. (Adapted from Queiroz, E.F., Wolfender, J.l., Atindehou, K.K., Traore, D., and Hostettmann, K., *J. Chromatogr. A*, 974(1–2), 123–134, 2002. With permission from Elsevier.)

12.4.1 Structural Investigation by Stop-Flow LC-NMR

As already mentioned, the use of LC-NMR in the stop-flow or the loop storage modes provides a more sensitive detection than in the on-flow mode, since more transients can be acquired on a given analyte. In stop-flow, the quality of solvent suppression is also enhanced, as shown in Figure 12.3. Furthermore, in this mode of operation, the acquisition of 2D NMR correlation experiments, often mandatory for complete structural investigation, is possible. Most studies use UV absorption to select peaks for stop-flow NMR acquisition, although MS signals can also be used.

An example of the improvement in S/N ratio obtained between the on-flow and stop-flow modes is shown in Figure 12.3 for the secoiridoid sweroside. The LC-NMR spectra of this compound were obtained directly from the analysis of crude extract of a Gentianaceae species of Africa, *Swertia calycina*. As shown, it was possible to obtain ^{1}H-^{1}H 2D correlation (COSY) directly from the LC peak in the crude extract in this mode. However, it is important to note that the quality of the spectra obtained in the stop-flow mode is lower than that of those obtained in the indirect hyphenation of NMR (see, for example, the CapNMR spectra of a similar compound [swertiamarin] in Figure 12.3d).

Stop-flow LC-NMR has been used for the study of numerous NPs, sometimes in combination with on-flow studies, or on a given peak identified to be of interest either by chemical LC-UV-MS screening or by on-line or at-line bioassays. Often, the same HPLC conditions as for on-flow measurements were used, but typically, the amounts of required extract were lower than for on-flow measurements.

Theoretically, all types of 2D NMR experiments can be acquired in stop-flow mode, provided that the sequences include solvent suppression for ^{1}H-^{1}H experiments. In most of the applications, the 2D spectra were mainly recorded in ^{1}H-^{1}H correlation such as COSY or TOCSY. Some applications, where 3D structural assignment was needed, have also used either rotating frame overhauser effect spectroscopy (ROESY) or nuclear overhauser enhanced spectroscopy (NOESY) experiments (Table 12.2).

In this respect, it has been noted that the absolute configuration determination is possible on-line by LC-CD 2D LC-ROESY and LC-TOCSY. This has been demonstrated for the on-line structural investigation of an isoquinoline and a naphthylisoquinoline alkaloid from the crude extract of a Dioncophyllaceae species *Habropetalum dawei* [92]. The absolute configuration was determined on-line by subsequent stop-flow LC-CD experiments on these compounds in the extract, and by empirical analysis of their CD data.

The acquisition of very sensitive ^{1}H-^{13}C correlation in the stop-flow mode, however, is difficult because of the relatively dilute sample analyzed. HSQC and HMBC have been successfully recorded in stop-flow, using concentrated fractions containing antifungal prenylated flavanones from Dipterocarpaceae *Monotes engleri* [22]. However, this has required a very high loading of the analyte in the flow cell.

Many examples of stop-flow and loop storage experiments have been described in the literature, either on crude extract or enriched fractions; they are summarized in Table 12.2. Often, the stop-flow experiments were triggered on given compounds of an extract. It has also been demonstrated that stop-flow experiments can be made at given time intervals ("time slicing") to reconstitute a type of pseudo LC-NMR on-flow run, but with many more transients in each increment. This was the case for the analysis of the crude extract of *Gnidia involucrata* (Thymelaceae), for which more than 20 benzophenones, xanthones, and flavone *O*- and *C*-glycosides were successfully detected [93].

Even if increased sensitivity is achieved in stop-flow LC-NMR or in loop storage modes, the need for solvent suppression restricts the available NMR detection window. This is illustrated by the measurement of stop-flow spectra of the pyrrolizidine alkaloid senecionine in two independent solvent systems, such as MeCN–D_2O and MeOH–D_2O. In the spectrum recorded in MeCN–D_2O, peaks corresponding to H-14a and H-6b were hidden by the suppression of the acetonitrile signal (Figure 12.8b). These resonances were, however, clearly visible when LC-NMR was performed in MeOH–D_2O, while H-5a and H-3b were, in this case, hidden by the solvent suppression of the MeOH signal (Figure 12.8a) [24]. This set of two spectra, together, provided complete information; however, this approach is not always practically applicable. Experiences in fully deuterated solvents

TABLE 12.2
Stop-Flow LC-NMR Applications for NP Identification

Products	Origin	Mode	Experiment	Instrumentation	Ref.
Lignans	*Torreya jackii*	C18 (250 × 4 mm ID, 5 μm), 300-μg inj., 0.8 mL/min	^{1}H, WET-COSY	500 MHz, 120-μL flow cell	[94]
α-Pyrones	*Ravensara crassifolia* (Mosher method)	C18 (100 × 8 mm ID, 10 μm), 10-mg inj., 0.8 mL/min	^{1}H	500 MHz, 60-μL flow cell	[95]
Naphthylisoquinoline alkaloids	*Ancistrocladus griffithii*	C18 (250 × 4.6 mm ID, 5 μm), 5-mg inj., 1.0 mL/min	^{1}H , TOCSY, ROESY	600 MHz, 120-μL flow cell	[43]
Lignans	Sesame-oil extracts	C18 (250 × 4.6 mm ID, 5 μm), 1-mg inj., 1 mL/min	^{1}H, stop-flow WET-TOCSY	600 MHz, 120-μL flow cell	[96]
Carotenoids	Thermally processed vegetables	C30 (250 × 4.6 mm ID, 5 μm), inj. N.A., 1 mL/min	^{1}H, WET-COSY	500 MHz, 60-μL flow cell	[97]
Phenolics	Cell culture of *Macleaya* and *Corydalis* species	C18 and OJ-RH (150 × 4.6 mm ID, 5 μm), inj. N.A., 1.0 mL/min	^{1}H, WET-NOESY	500 MHz, 60-μL flow cell	[98]
Roquefortine (toxins)	*Penicillium roqueforti*, *P. paneum*, and *P. crustosum*	C12 (250 × 4.6 mm ID, 5 μm), inj. N.A., 1 mL/min	^{1}H	600 MHz, 60-μL flow cell, cryoflow probe	[99]
Iridoid glycosides	*Jamesbrittenia fodina*	C18 (250 × 8 mm ID, 5 μm), 200-μg inj., 1 mL/min	^{1}H	500 MHz, 60-μL flow cell	[85,86]
Isoflavones	*Radix astragali*	Cap-LC-NMR, C18 (150 × 0.25 mm ID, 5 μm), 33-μg inj., 5 μL/min	^{1}H, fully deuterated solvent CD_3CN-D_2O	600 MHz, 5-μL solenoidal flow cell	[100]
Flavonoids, phenols	*Blumea gariepina*	C18 (100 × 8 mm ID, 10 μm), 10-mg inj., 1.0 mL/min	^{1}H	500 MHz, 60-μL flow cell	[82]
Phenylphenalenone derivatives	*Xiphidium caeruleum*	C18 (250 × 4 mm ID, 5 μm), inj. N.A., 0.8 mL/min	^{1}H	500 MHz, 120-μL flow cell	[101]

(*Continued*)

TABLE 12.2 (CONTINUED)
Stop-Flow LC-NMR Applications for NP Identification

Products	Origin	Mode	Experiment	Instrumentation	Ref.
Flavonoids	*Citrus sinensis*	C18 (250 × 4.6 mm ID, 5 µm), inj. N.A., 0.2 mL/min	^{1}H	400 MHz, 60-µL flow cell	[102]
Tetrahydroisoquinolines, alkaloids	*Corydalis* and *Macleaya* spp. cell cultures	C18 and OJ-RH (150 × 4.6 mm ID, 5 µm), inj. N.A., 1.0 mL/min	^{1}H	500 MHz, 60-µL flow cell	[103]
Alkaloids	*Nandina domestica*	C18 and OJ-RH (150 × 4.6 mm ID, 5 µm), inj. N.A., 1.0 mL/min	^{1}H	500 MHz, 60-µL flow cell	[104]
Stilbenes, phloroglucinols, flavonoids	*Lysidice brevicalyx*	C18 (150 × 4.6 mm ID, 5 µm), inj. N.A., 0.8 mL/min	^{1}H , COSY	500 MHz, 60-µL flow cell	[105]
Flavonoids, cinnamic acids, coumarins	*Chamomilla recutita*	C18 (250 × 4.6 mm ID, 5 µm), 5-mg inj., 1.0 mL/min	^{1}H , Loop storage	400 MHz, 60-µL flow cell	[106]

N.A., Not available.

Note: WET: Water suppression Enhanced through T1 effects; COSY: COrrelation SpectrocopY; ROESY: Rotating frame Overhauser Effect SpectroscopY; TOCSY: TOtal Correlation SpectrocopY; NOESY: Nuclear Overhauser Enhanced Spectroscopy.

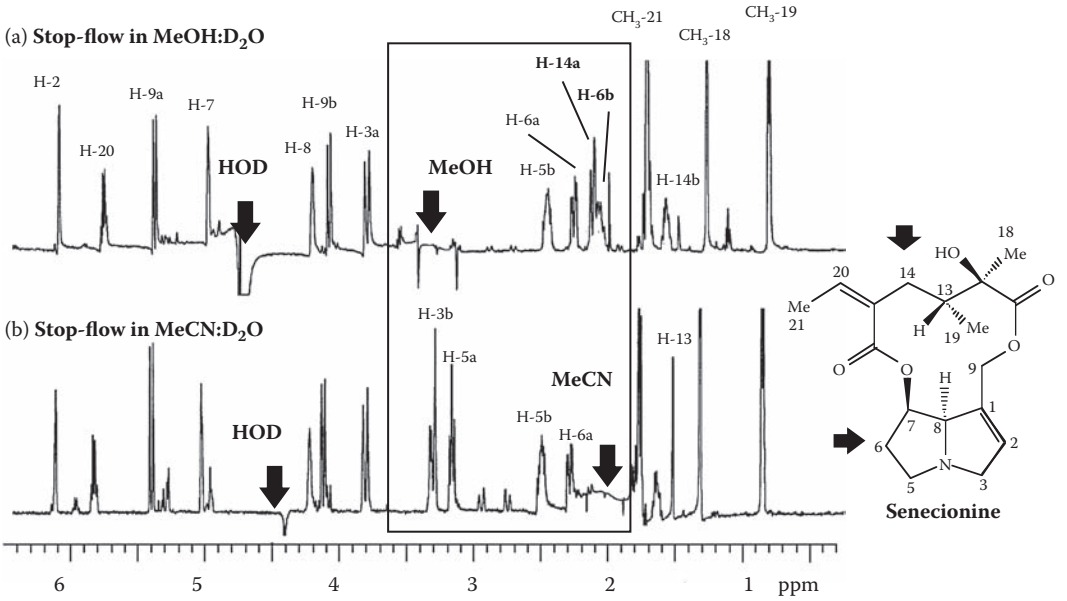

FIGURE 12.8 Stop-flow spectra measured for senecionine in the lipophilic extract of *Senecio vulgaris* (Asteraceae) in both MeCN–D_2O and MeOH–D_2O solvent systems. HPLC conditions: Inj. 3 mg; Col. Symmetry C-18 (150 × 3.9 mm ID, 5 μm); (a) MeOH–D_2O gradient (10:90 to 70:30 in 30 min); (b) MeCN–D_2O gradient (5:95 to 50:50 in 20 min); 0.015 M NH_3; 0.8 mL/min. LC-NMR conditions: flow cell (60 μL, 3 mm ID), 500 MHz. (MeCN: acetonitrile; D_2O: deuterated water; MeOH: methanol.) (Adapted from Wolfender, J.l., Ndjoko, K., and Hostettmann, K., J. *Chromatogr. A,* 1000(1–2), 437–455, 2003. With permission from Elsevier.)

or with superheated D_2O have also been reported, and in this case, the solvent-suppression issue was satisfactorily overcome.

12.4.2 Applications of At-Line LC-SPE-NMR to Crude Plant Analysis

As described, the use of indirect hyphenation of LC-NMR represents an interesting alternative to overcome the limitations of direct hyphenation. In this respect, since its introduction about one decade ago, HPLC-SPE-NMR has been widely used in the field of NP analysis, and most of the recent studies have been performed with this method, since fully automated systems are commercially available. In most of the applications, rather small quantities of extract were injected, and sufficient amounts of analytes were obtained by multiple trapping of the peaks of interest.

In most of the applications, CD_3CN and CD_3OD have been used to elute the analytes from the SPE, and usually less than 1 mL of deuterated solvent was needed per sample. Contrary to stop-flow spectra, and because of the sample concentration increase obtained by multiple trapping, 2D ^1H-^{13}C HSQC and HMBC spectra were acquired for most of the major peaks of the extracts analyzed, while sensitive ^1H-NMR spectra were obtained only for the minor constituents.

The first study in the field of NPs is relatively recent, appearing in 2003, and used automated LC-SPE-NMR for the identification of flavonoids, rosmarinic acid, and carvacrol in a crude lipophilic extract of oregano, with the use of a LC-SPE-NMR cryogenic probe [35]. The extract loading was 1.2 mg (150 × 4.6 mm ID C18 column), and 2D ^1H-^{13}C NMR were obtained on the six main constituents after triple trapping. Interestingly, since CD_3CN was used as the solvent to elute the peaks from the cartridges, exchangeable protons, such as the chelated OH of flavonoids, were readily observable; this would not have been possible in standard LC-NMR conditions. Since then, many applications of SPE-NMR have been reported for the rapid identification of NPs in various crude extracts. They are summarized in Table 12.3.

TABLE 12.3
SPE-NMR Applications for NP Identification

Products	Origin	Remarks	Experiment	Instrumentation	Ref.
Phenolics	Oregano	Hysphere GP / CD_3CN	^1H, ^{13}C, TOCSY, HSQC, HMBC	400 MHz, 120-μL flow cell 600 MHz, 20-μL flow cell (cryoprobe)	[35]
Saponins	*Quillaja saponaria*	Hysphere GP / CD_3OD (off-line enrichment)	^1H	600 MHz, 120-μL flow cell	[107]
Flavonoids and cardenolides	*Kanahia laniflora*	Hysphere C18 HD / CD_3CN	^1H, COSY NOESY, HSQC, HMBC	600 MHz, 30-μL flow cell	[108]
Phenolics	Olive oil	Hysphere / CD_3CN	^1H, 1D NOESY, TOCSY	600 MHz, 30-μL flow cell	[109]
Flavonoids, chorogenic acids	*Rhaponticum carthamoides*	Hysphere SH / CD_3CN, CD_3OD	^1H, HMBC	400 MHz, 120-μL flow cell	[110]
Iridoid glycosides	*Harpagophyton procumbens*	Hysphere GP or C18 / CD_3CN	^1H	600 MHz, 30-μL flow cell	[111]
Isoflavonoids	*Smirnovia iranica*	Hysphere C18 HD / CD_3CN	^1H, ^{13}C DEPT, COSY, NOESY, HSQC, HMBC	600 MHz, 30-μL flow cell	[32]
Diterpenes	Rosemary extract	Hysphere SH / CD_3OD $CDCl_3$	^1H, COSY, TOCSY, HSQC, HMBC	400 MHz, 120-μL flow cell	[112]
Lignans	*Phyllanthus urinaria*	Hysphere GP / CD_3CN	^1H	400 MHz, 120-μL flow cell	[113]
Phenolics	*Tilia europea*, *Urtica dioica*, *Lonicera periclymenum*, and *Hypericum perforatum*	Hysphere GP / CD_3CN	^1H, 1D-NOESY	400 MHz, 120-μL flow cell 600 MHz, 20-μL flow cell (cryoprobe)	[114]
Isomeric tropane alkaloids	*Schizanthus grohamii*	Hysphere GP / CD_3CN CD_3OD LC-NMR, stop-flow, loop storage	^1H, COSY	600 MHz, 30-μL flow cell (cryoprobe)	[115]
Chinane-type tricyclic diterpenes	*Harpagophytum procumbens*	Hysphere GP / CD_3CN	^1H, COSY, NOESY, HSQC, HMBC	600 MHz, 30-μL flow cell	[116]
Diterpenes, iridoids, phenolics	*Harpagophytum procumbens*	Hysphere GP / CD_3CN	^1H, COSY, NOESY, HSQC, HMBC	600 MHz, 30-μL flow cell	[117]

Polyacetylenes	Pure products (Mosher method)	Hysphere GP / $CDCl_3$	^1H	500 MHz, 60-µL flow cell	[118]
Alkaloids	*Corydalis solida*	Hysphere GP / CD_3OD	^1H, COSY, HSQC, HMBC	600 MHz, 30-µL flow cell (cryoprobe)	[36]
Sesquiterpenes	*Penicillium roqueforti*	Hysphere GP / CD_3CN dissolve in C_6D_6 and analysis in 3-mm tubes	^1H, COSY, NOESY, HSQC, HMBC	600 MHz, 3-mm tubes	[119]
Flavonoids, phenolics, phloroglucinols	*Hypericum perforatum*	Hysphere GP / CD_3CN	^1H, COSY, TOCSY	400 MHz, 120-µL flow cell	[120]
Phenolics	*Phyllanthus reticulatus*	Hysphere GP / CD_3CN	^1H	400 MHz, 120-µL flow cell	[121]
Sesquiterpenes	*Warburgia salutaris*	Hysphere GP / CD_3CN	^1H, COSY, HMBC, HSQC	600 MHz, 30-µL flow cell	[122]
Flavonoid glycosides	*Neolitsea sericea*	Hysphere GP / CD_3CN	^1H	400 MHz, 30-µL flow cell	[123]
Flavonoid glycosides acylated	*Machilus philippinensis*	Hysphere GP / CD_3CN	^1H, COSY, NOESY	400 MHz, 30-µL flow cell	[124]
Gossypol	*Thespesia danis*	Hysphere GP / CD_3CN combined with CD	^1H	600 MHz, 30-µL flow cell	[59]

N.A., Not available. In remarks, only the type of cartridges used and the solvent for transfer are mentioned; most of the separations are carried out in standard reversed-phase HPLC conditions

Note: COSY: COrrelation SpectrocopY; ROESY: Rotating frame Overhauser Effect SpectroscopY; TOCSY: TOtal Correlation SpectrocopY; NOESY: Nuclear Overhauser Enhanced SpectroscopY; HSQC: Heteronuclear Single Quantum Coherence; HMBC: Heteronuclear Multiple Bond Coherence; CD_3CN: deuterated acetonitrile; CD_3OD: deuterated methanol.

The method has also been used in combination with bioassay and, for example, with the on-line detection of antioxidants [110].

In order to illustrate the type of data that can be obtained with this approach, the results obtained with an approach combining SPE trapping and the mass sensitivity detection of a CapNMR are described in more detail [33]. LC-SPE-CapNMR was used for the rapid identification of complex sesquiterpene lactones and esterified phenylpropanoids present in an enriched ethanolic extract of *Thapsia garganica* fruits. The extract was separated with a reversed-phase two-step linear MeCN–H_2O gradient, showing the presence of numerous constituents of the toluene fraction (Figure 12.9a). Twelve well-resolved peaks were selected for the HPLC-SPE trapping, based on the absorbance thresholds defined at 230 nm on an automated device that handles trays with 96 cartridges. In order to match with the low active volume of the CapNMR probe, 10 × 1 mm ID SPE cartridges were used instead of 10 × 2 mm ID cartridges. Indeed, experiments performed on a pure standard revealed that the elution profiles of the 10 × 1 mm ID SPE cartridges filled with poly(divinylbenzene) resin were similar to those obtained by direct injection into the CapNMR probe; they also matched its

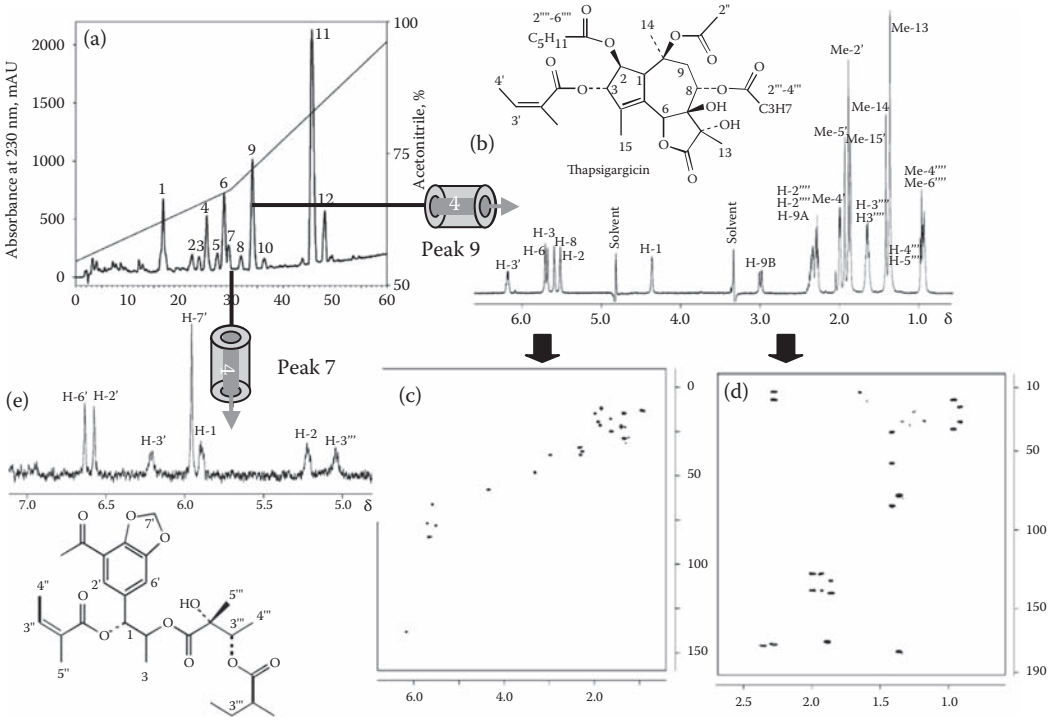

FIGURE 12.9 (a) HPLC trace of the toluene fraction of ethanolic extract of *Thapsia garganica* fruits. Peaks selected for HPLC–solid-phase extraction (SPE) trappings are labeled 1–12. HPLC conditions: Inj. 0.31 mg; Col. Phenomenex Luna C18 (150 × 4.6 mm ID, 5 µm), MeCN–H_2O gradient, flow rate 0.5 mL/min, 40°C. SPE-NMR trapping on Hysphere GP (10 × 1 mm ID), eluted with CD_3OD, flow cell capillary nuclear magnetic resonance (CapNMR) 5 µL (Protasis/MRM Corp., Savoy, Il.); 500 MHz. (b) ^{1}H-NMR spectrum (NT: 256) acquired for peak 9 containing thapsigargicin. Shown is a difference spectrum obtained by subtraction of a spectrum obtained by eluting an empty cartridge from the actual spectrum in order to eliminate solvent resonances (both spectra were acquired without solvent peak suppression). (c) Heteronuclear Single Quantum Coherence (HSQC) spectrum acquired with peak 11 containing thapsigargin; 4 transients, 512 increments, total acquisition time 43 min. (d) Aliphatic region of an Heteronuclear Multiple Bond Coherence (HMBC) spectrum acquired with peak 11; 24 transients, 512 increments, total acquisition time 4 h. (e) ^{1}H-NMR spectrum (NT: 2000) acquired for the minor peak 7. (Adapted from Lambert, M., Wolfender, J.l., Staerk, D., Christensen, B., Hostettmann, K., and Jaroszewski, J.W., *Anal. Chem.*, 79(2), 727–735, 2007. With permission from the American Chemical Society.)

filling volume (5 μL) [33]. A postcolumn make-up flow of water was added to the eluate in a ratio of 1:2 in order to improve retention of compounds on the cartridges. Four cumulative trappings, with 0.31 mg of the extract per injection, were performed. After drying, the cartridges were eluted, and the eluate was transferred to the CapNMR probe. Parallel LC-MS measurements in positive, as well as in negative ion mode, were performed in order to assist with structure elucidation.

Using this technique, nine NPs present in the plant extract, in amounts varying from 0.1% to 20%, were identified by means of 1D and 2D NMR spectra and LC-MS measurements. Examples of some of the ^{1}H-NMR spectra obtained with LC-SPE-CapNMR are shown in Figure 12.9. Excellent-quality 2D spectra were obtained rapidly with the more abundant extract constituents, including the least sensitive experiments such as HMBC and NOESY. Good or satisfactory 1D ^{1}H-NMR spectra were obtained with minor extract constituents (Figure 12.9e).

^{1}H-NMR spectra of peaks 1, 4, 5, and 8 were characteristic of polyoxygenated guaianolides, found in the genus *Thapsia* [125]. The ^{1}H-NMR spectrum recorded showed that the main peak 9 (Figure 12.9b) corresponded to thapsigargicin. COSY, TOCSY, HSQC (Figure 12.9c), and HMBC (Figure 12.9d) spectra allowed assignments in agreement with literature data [126]. The signals characteristic of the thapsigargicin skeleton were mainly five methine hydrogens at δ 4.36 (H-1), 5.51 (H-2), 5.59 (H-8), 5.67 (H-3), and 5.71 (H-6), and a pair of diastereotopic methylene hydrogens at δ 2.31 (H-9A) and 2.99 (H-9B). Three methyl group signals at δ 1.36 (H-13), 1.41 (H-14), and 1.86 (H-15) were observed. The remaining resonances found in the ^{1}H-NMR spectrum belong to the angelic acid residue and to aliphatic acyl side chains attached to O-2, O-8, and O-10. The presence of thapsigargicin in peak 9 was in agreement with negative ion mode ESIMS (m/z = 621.3, $[M–H]^-$). Comparison with a pure standard shows that a single injection of the extract (0.31 mg) corresponds to about 70 μg (110 nmol) of thapsigargicin.

The advantage of HPLC-SPE combined with CapNMR is even more apparent in the case of the penylpropanoids (peak 6, 7, 10, 12). Using a reference standard for comparison, the amount of the minor phenylpropanoids derivative (peak 7) was roughly 2 μg (4 nmol) per injection, or 0.6% of the toluene extract (0.02% of the fruits), and usable ^{1}H-NMR spectra were obtained (Figure 12.9e). The amount of peak 10 was estimated at 0.4 μg per injection (0.7 nmol) or 0.1% of the extract (0.003% of the fruits). A sufficient amount of this compound could nevertheless be accumulated in the HPLC-SPE step, leading to the elucidation of its structure. In this example, with the exception of the absolute configuration, the complete structural assignments of nine NPs, constituting less than 0.5% of the extract, were successfully performed [33].

12.4.3 Application of At-Line CapNMR

As shown earlier, CapNMR can be successfully used in the SPE-NMR mode, but this method is mainly used for the analysis of dried LC peaks collected in 96-well plates or in Eppendorf tubes in the indirect hyphenation with HPLC [9,27]. The method has also been reported recently to be efficiently implemented in a high-throughput NP chemistry platform for the generation and analysis of large NP libraries [127]. In this respect, many different applications to NPs have been reported over the last four years, since a probe (CapNMR™) has become commercially available for most of the existing NMR systems.

In 2002, a seminal application of the technique to the NP field was reported by Eldrige et al., who demonstrated that a library of flash fractions containing one to five compounds from the stem bark of *Taxus brevifolia* was successfully dereplicated by acquisition of ^{1}H and COSY spectra, using amounts as small as 5 μg [128].

In most of the applications reported since then, both in the plant and animal fields, CapNMR enabled the acquisition not only of high-quality ^{1}H-NMR spectra but often of a full set of 2D spectra, including HSQC and HMBC experiments, for the unambiguous structure determination of NPs at-line. Some of these recent applications are reported in Table 12.4.

Besides applications to crude extracts, which demonstrated that the quality of NMR data obtained at-line enabled either dereplication or full identification of novel compounds, different applications

TABLE 12.4
CapNMR Applications for NP Identification

Products	Origin	Remarks	Experiment	Instrumentation	Ref.
Alkaloids	*Taxus brevifolia*	Creation of a library, flash chromatography fractions	^{1}H, COSY, HMBC, HSQC	600 MHz, 5-μL solenoidal flow cell	[128]
Terpenoid mixture	Pure products	Capillary HPLC, C18 (150 × 8 mm ID, 5 μm), 3 μL/min, 2 cpds on-flow, 10-mM concentration	^{1}H	500 MHz, 1.1-μL solenoidal flow cell (homemade)	[134]
Cardenolides and steroids	*Lucidota atra*	13 steroids from 50 fireflies	^{1}H, COSY, NOESY, HSQC, HMBC	600 MHz, 5-μL solenoidal flow cell	[131]
Cyclolignans	*Scyphocephalium ochocoa*	C18 (250 × 8 mm ID, 5 μm) 50 mg of extract, 2 cpds	^{1}H, COSY, NOESY, HSQC, HMBC	600 MHz, 5-μL solenoidal flow cell	[135]
Acylated caprylic alcohol glycosides	*Arctostaphylos pumila*		N. A.	600 MHz, 5-μL solenoidal flow cell	[37]
Indolosesquiterpene	*Greenwayodendron suaveolens*	C18 (250 × 8 mm ID, 5 μm), 50 mg of fraction, 1 cpd, 300 μg	^{1}H, COSY, NOESY, HSQC, HMBC	600 MHz, 5-μL solenoidal flow cell	[136]
Iridoid glycosides	*Penstemon centranthifolius*,	C18 (250 × 8 mm ID, 5 μm), 50 mg of fraction, 6 cpds, 25–300 μg of material	^{1}H, COSY, NOESY, HSQC, HMBC	600 MHz, 5-μL solenoidal flow cell	[27]
Isoflavones	*Radix astragali*	Capillary HPLC, on-flow, 33 μg of extract injected, 4 cpds	^{1}H	600 MHz, 5-μL solenoidal flow cell	[100]
Acetylated oligorhamnosides	*Cleistopholis patens*	C18 (250 × 8 mm ID, 5 μm), 50 mg of fraction, 2 cpds, 70 and 200 μg	^{1}H, ^{13}C, COSY, NOESY, HSQC, HMBC	600 MHz, 5-μL solenoidal flow cell	[137]
Ursene triterpenes	*Diospyros dendo*	C18 (250 × 8 mm ID, 5 μm), 35 mg of fraction, 5 cpds, 3–20 μg	^{1}H, COSY	600 MHz, 5-μL solenoidal flow cell	[138]
Isoflavone	*Psorothamnus arborescens*	C18 (250 × 8 mm ID, 5 μm), 50 mg of fraction, 2 cpds, 150 μg	^{1}H, COSY, NOESY, HSQC, HMBC	600 MHz, 5-μL solenoidal flow cell	[139]
Oligosaccharides	Heparin	Coupling capillary isotachophoresis (cITP)	^{1}H, COSY, TOCSY, ROESY	600 MHz, homemade solenoidal flow cell	[140]
Sesquiterpene lactones esterified phenylpropanoids	*Thapsia garganica*	SPE-CapNMR, Hysphere GP, C18 (150 × 4.6 mm ID, 3 μm), 0.3 mg, 4 trappings, 9 cpds	^{1}H, COSY, NOESY, HSQC, HMBC	500 MHz, 5-μL solenoidal flow cell	[33]
Lignan	*Justicia laxa*		N. A.	600 MHz, 5-μL solenoidal flow cell	[141]

Phomosine A	Fungal or bacterial extracts *Muehlenbeckia* sp. and *Aspergillus* sp.	Dereplication by search in databases, 400–700-µg extracts, ca. 20 µg per cpds	^{1}H	500 MHz, 5-µL solenoidal flow cell	[142]
Peptaibol	Mycoparasitic fungus *Sepedonium chrysospermum*	C18 (N.A.) 700-µg inj., 1 cpd 33 µg, high MW: 1619	^{1}H, COSY	500 MHz, 5-µL solenoidal flow cell	[143]
Indolic alkaloids	Cyanobacterial extract *Fischerella ambigua*	C18 (250 × 4.6 mm ID, 5 µm) 30-µg inj.	^{1}H	500 MHz, 5-µL solenoidal flow cell	[39]
Oxylipin (wound-induced)	*Arabidopsis thaliana*	C18 (500 × 10 mm ID, 5 µm) high resolution separation	^{1}H, COSY, TOCSY, HSQC, HMBC	500 MHz, 5-µL solenoidal flow cell	[17,132]
Mycolaexins	Fungal confrontation *Eutypa lata* and *Botryosphaeria obtusa*	C18 (250 × 10 mm ID, 5 µm), 40-mg inj.	^{1}H, COSY, TOCSY, NOESY, HSQC, HMBC	500 MHz, 5-µL solenoidal flow cell	[133]

N.A., Not available.

Note: COSY: COrrelation SpectrocopY; TOCSY: TOtal Correlation SpectrocopY; NOESY: Nuclear Overhauser Enhanced SpectroscopY; HSQC: Heteronuclear Single Quantum Coherence; HMBC: Heteronuclear Multiple Bond Coherence.

with limited biological samples were also reported. Some examples include the analysis of spider venoms [129,130], of steroids from a few fireflies [131], of induced plant defense hormones [17,132], or the study of fungal metabolites at the Petri dish scale [133].

A new field in which sensitive at-line NMR approaches, such as CapNMR, are starting to play an important role is that of plant metabolomics. In this field, conventional NMR methods have been widely used for the rapid and direct profiling of extracts and mainly reveal major biomarkers both in biological fluid analysis [144] and in plant studies [145]. In some biological interactions, however, subtle variations of the metabolome occur, in which minor biomarkers may play a key role. For the detection of these low-abundance compounds, NMR-based approaches are limited. In this field and, for example, in the study of plant response to stress, MS-based metabolomics are playing an increasingly important role [146,147].

MS-based metabolomic strategies alone are often not sufficient for the full identification of new biomarkers. In this respect, CapNMR used at-line represents a key strategic tool, since the biomarkers are clearly sample-limited. The MS-based metabolomics results provide a precise localization of the biomarkers of interest in the HPLC profiles, and LC-MS-triggered microfractionation of the relevant metabolites in a given biological interaction can be undertaken for further NMR characterization [17].

This is illustrated by the application of CapNMR for the identification of original jasmonate derivatives related to the wound response in the model plant *Arabidopsis thaliana* (Brassicaceae). In this selected example, significant metabolome variations related to stress caused by wounding were studied by an ultra performance liquid chromatography–time-of-flight mass spectrometry (UPLC-TOF-MS) metabolomic approach [148]. Extensive research on the wound response has indeed brought to light important information regarding the plant defense mechanism against herbivores, and different bioactive oxygenated fatty acids belonging to the jasmonate family were found to be responsible for the expression of defense genes [149]. UPLC-TOF-MS has permitted the detection of significant minor wound-biomarker peaks among major constitutive metabolites. Besides known signaling molecules, original oxylipins and related products were highlighted by this approach [148,150].

A targeted LC-MS-triggered microfractionation of these compounds at the semipreparative level was undertaken for the identification of the wound biomarkers at-line, using CapNMR. Due to the complexity of plant extracts, the purification of these metabolites, present in very low concentrations, was found to be critical. The strategy used for purification relied on the optimization of the chromatographic conditions at the analytical level, using UPLC-TOF-MS, owing to modeling software. The optimized method was then transferred to semipreparative LC-MS.

As shown in Figure 12.10a, the UPLC-TOF-MS analysis performed on a long column provided a very detailed metabolite profiling of *A. thaliana* constituents. A thorough multivariate analysis of metabolite fingerprinting results obtained on many extracts of wounded and unwounded *A. thaliana* leaves revealed several key wound biomarkers with characteristic molecular ions (Figure 12.10b) [148]. In Figure 12.10b, *m/z* 209 was identified as the well-known plant hormone jasmonic acid (JA) and *m/z* 417 as its glucoside derivative. Other biomarkers, such as the ion *m/z* 225, were found to be clearly induced after wounding (Figure 12.10c) and exhibited four different isomeric forms. The mass difference with JA (16 Da) revealed a possible hydroxylation. However, since four isomers were present, the exact hydroxylation position could not be ascertained by MS alone.

The extraction of a thousand wounded specimens of *A. thaliana* provided sufficient material for microisolation of very minor wound biomarkers and their characterization by CapNMR. Optimal separation conditions were predicted and tested at the analytical scale using UPLC-TOF-MS. Two preliminary analyses of the extract with different gradient slopes were necessary to model the retention of each compound and find appropriate conditions for an optimal separation (i.e., in isocratic and/or gradient modes) [151]. The biomarkers of interest were localized in each chromatogram according to their deprotonated molecular ions $[M\text{-}H]^-$. Examples of the latter are given in Figure 12.10b (*m/z* 225, 417, and 209).

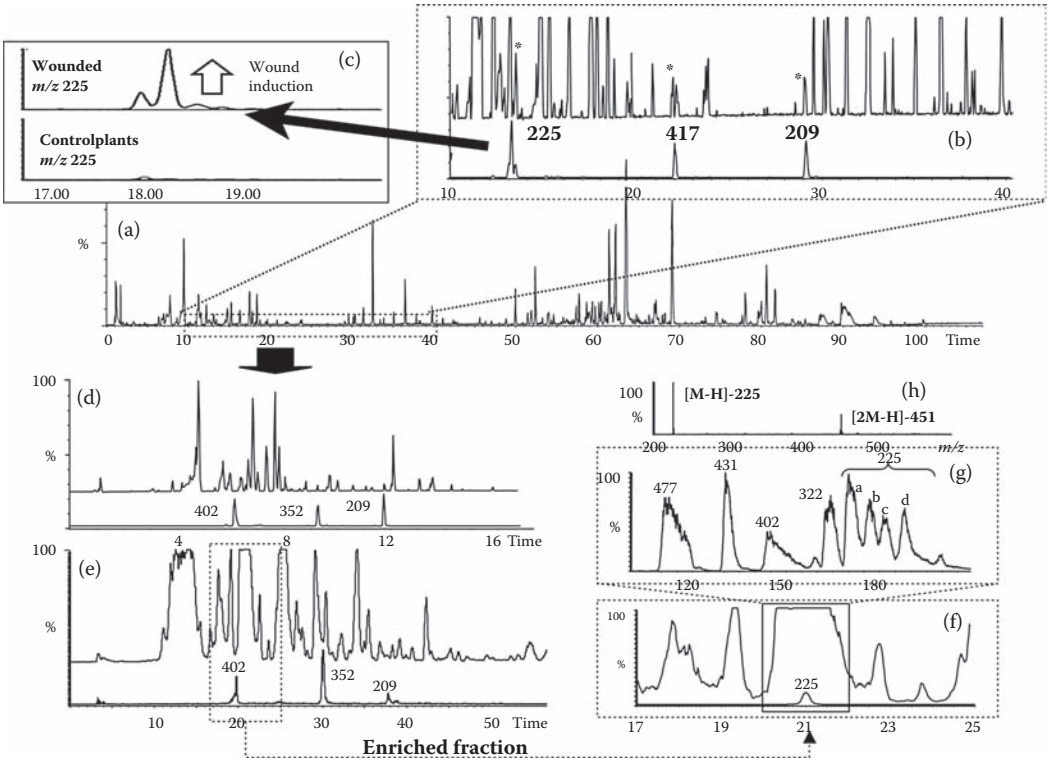

FIGURE 12.10 Scheme of the general strategy used for the targeted microisolation of wound biomarkers in *Arabidopsis thaliana*. (a) Ultra performance liquid chromatography–time-of-flight–mass spectrometry (UPLC-TOF-MS) analyses of the isopropanol extract of wounded *A. thaliana* leaves. Base peak ion (BPI) traces (*m/z* 100–1000) in the electrospray negative ion mode are presented. 1% per minute slope acidic acetonitrile–water gradient. (b) Zoom in the region containing some of the wound-induced metabolites of interest and sum of their selected ion traces. (c) Comparison of the extracted ion traces of the wound biomarker *m/z* 225 showing the induction of four distinct isomers after leaf wounding. (d) Transfer from UPLC to semipreparative LC and isolation of the *m/z* 225 isomers. (e) Semipreparative LC transferred chromatogram and sum of marker traces. (f) Zoom in the region containing *m/z* 225 isomers (highlighted by the single ion trace) and selection of the fraction (RT 20–22 min). (g) Isocratic semipreparative chromatogram. (h) MS spectrum summed on all *m/z* 225 isomers showing no interference with other coeluting compounds. (Adapted from Glauser, G., Guillarme, D., Grata, E., Boccard, J., Thiocone, A., Carrupt, P.A., Veuthey, J.l., Rudaz, S., and Wolfender, J.l., *J. Chromatogr. A*, 1180, 90–98, 2008. With permission from Elsevier.)

This chromatographic modeling revealed that a relatively high efficiency (>30,000 plates) was mandatory to ensure satisfactory peak purity for further CapNMR analysis. This purity could be reached only with a long semipreparative column length (500 mm) at isocratic conditions. To ensure a satisfactory purification of minor biomarkers, a prefractionation step of the extract in gradient mode was thus mandatory before applying these high-resolution isocratic conditions. This prefractionation step (Figure 12.10d) was precisely computed by applying chromatographic transfer from UPLC, based on reported equations [152,153], and providing enriched simplified fractions containing few compounds with close hydrophobic properties (Figure 12.10e and f).

The microfraction containing the mixture of *m/z* 225 biomarkers is shown in Figure 12.10f. Based on the computed optimized isocratic condition, this enriched fraction was finally separated on two C18 columns (both 250 × 10 mm ID, 5 μm), providing 35,000 96-well plates. The results of the separation are shown in Figure 12.10g and reveal an overall separation of all constituents and a satisfactory resolution of the *m/z* 225 isomers. The MS spectrum demonstrated the absence of coeluting ions

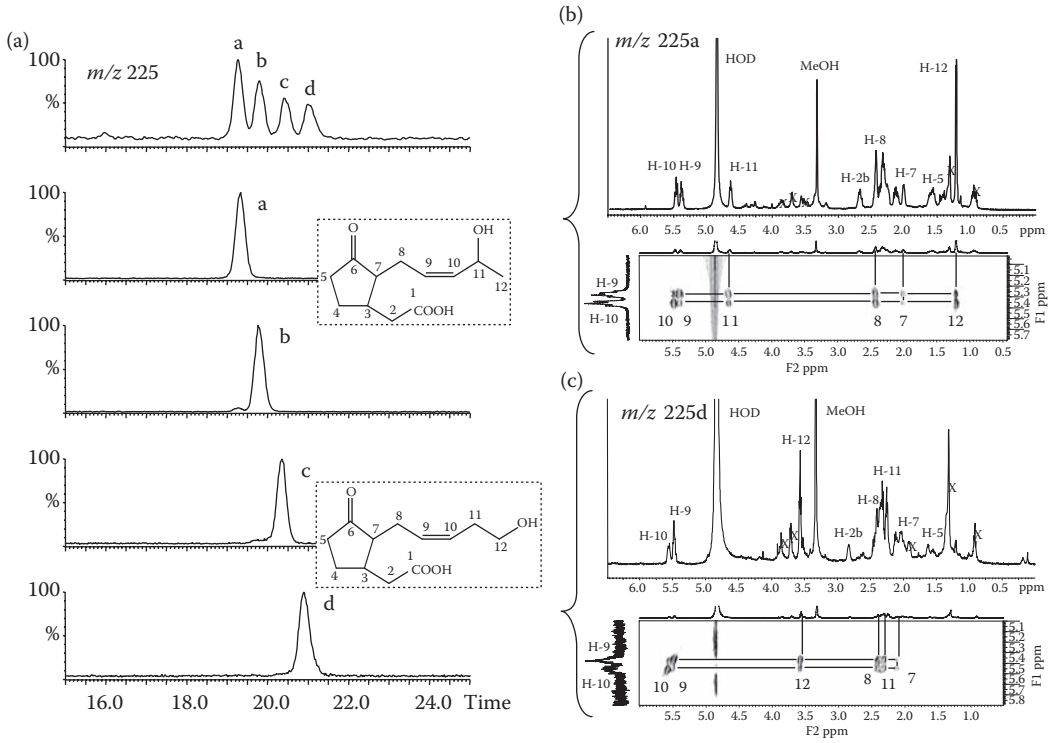

FIGURE 12.11 (a) Isocratic ultra performance liquid chromatography–time-of-flight–mass spectrometry (UPLC-TOF-MS) total ion current (TIC) chromatograms of the *m/z* 225 isomer mixture and of the different isolated compounds *m/z* 225a, 225b, 225c, and 225d. (b) Capillary nuclear magnetic resonance (CapNMR) and TOtal Correlation SpectroscopY (TOCSY) correlations of H-9 and H-10 for *m/z* 225a. (c) CapNMR and TOCSY correlations of H-9 and H-10 for *m/z* 225d. (Adapted from Glauser, G., Guillarme, D., Grata, E., Boccard, J., Thiocone, A., Carrupt, P.A., Veuthey, J.l., Rudaz, S., and Wolfender, J.l., *J. Chromatogr. A*, 1180, 90–98, 2008. With permission from Elsevier.)

(Figure 12.10h). Although *m/z* 225 peaks were not baseline resolved (Rs_{min} 0.8), the collection was precise enough to pool pure microfractions totaling more than 80% of each peak for further NMR investigation, with a fractionation performed every 30 seconds in 96-well plates. Each peak was successfully isolated to the level of a few tenths of a microgram, without interfering compounds. Figure 12.11a shows the mixture of the four isomers and the isolated fraction of each isomer. Each *m/z* 225 isomer was dissolved in 5 µL of CD_3OD and parked into the CapNMR probe with a 9 µL push volume.

Their exact deprotonated molecular mass *m/z* 225.1127 ($C_{12}H_{17}O_4$), measured by TOF-MS, indicated that the four isomers could be hydroxylated jasmonates. 12-Hydroxyjasmonate (12-HOJA), also known as tuberonic acid, has been described to occur naturally in *A. thaliana* [154]. A comparison of the NMR spectrum of *m/z* 225d (Figure 12.11c) with that published for tuberonic acid [155] demonstrated that both spectra were identical, despite small impurities coming from the 96-well plates and the stationary phase. The structure was further confirmed by 2D NMR. In particular, a 2D TOCSY experiment (Figure 12.11c) enabled the isolation of the spin system of the lateral hydroxylated side chain (H-7 to H-12). It revealed a clear correlation between the olefinic H-10 (δ 5.48), a methylene H-11 (δ 2.31), and a terminal primary alcohol group H-12ab (δ 3.55). This proved the hydroxylation position of the side chain in 12 (Figure 12.11c). The peak *m/z* 225c showed the same typical correlation. Selective decoupling performed by irradiation of methylene H-11 (δ 2.31) and H-8 (δ 2.39) in both *m/z* 225c and 225d, demonstrated that the double bond was in a (*Z*) configuration (H-9/H-10 coupling constant, $J = 11$ Hz). Therefore, the only possible difference

between both isomers was an epimerization of the cyclopentanone from a *cis* to *trans* configuration, which was confirmed by the shift of methylene signal H-2b (δ 2.81) in *m/z* 225c compared to that of 225d (δ 2.62) [156]. This type of epimerization at C-7 has been reported elsewhere for JA and has a strong effect on plant response [157].

The first two eluting isomers, *m/z* 225a and 225b, showed very similar NMR spectra, closely related to those of *m/z* 225c and 225d. A TOCSY experiment demonstrated a clear correlation between olefinic H-10 (δ 5.44), a secondary alcohol group H-11 (δ 4.62), and a terminal methyl group H-12 (δ 1.19) (Figure 12.11b). This indicated a modification of hydroxylation on the side chain of *m/z* 225a/b. Therefore, the two additional polar isomers were identified as 11-hydroxyderivatives of JA (11-HOJA). Both possessed a (*Z*) double bond, and the major difference was likely due to an epimerization at C-7 of the cyclopentanone ring, since a shift of methylene H-2b was measured. These original wound biomarkers have been found to be putative clearance metabolites of JA [150], and their de novo structure identification would not have been possible without the at-line use of sensitive CapNMR.

As shown here, because of the convoluted nature of plant extracts, the at-line NMR identification of key biomarkers in very low concentrations represents a challenging task. This study demonstrated that a thorough HPLC separation strategy prior to CapNMR measurement is mandatory; the developed generic LC-MS-monitored microisolation procedure [17] provided microgram amounts of biomarkers with a satisfactory purity for unambiguous isomer discrimination.

12.4.4 Detection without HPLC Separation

In the different examples discussed, the importance of HPLC prior to NMR detection has been stressed, and the last example clearly demonstrates that chromatography is crucial for the identification of very minor metabolites. When dealing with more abundant secondary metabolites, HPLC separation is not always mandatory for identification. NMR itself can be considered both as a profiling method when a crude extract is analyzed and as an identification method when a set of signals is either correlated in the form of 2D experiments or compared to a database. Direct NMR profiling of a crude extract is more commonly used for a rapid evaluation of extract composition, especially in the quality control of herbal medical products and metabolomics [158]. For example, for the quality control of gingko, ginseng, and *Hypericum* phytopreparations, alternative methods that do not require HPLC separation have been reported [8].

In the case of *Ginkgo biloba*, the determination of the content in allergenic ginkgolic acids has been evaluated by direct ^{1}H-NMR analysis of various commercial preparations without any prepurification steps. The aromatic protons common to all of these compounds were well separated from other signals in the total ^{1}H-NMR spectra of the extracts. The quantity of the compounds was calculated from the relative ratio of the integral of these NMR peaks with an internal standard, and the results were comparable with other chromatographic methods of quantification [159]. Similarly for *Hypericum perforatum*, hypericin and its derivative pseudohypericin were efficiently quantified by ^{1}H-NMR, with the specific detection of strongly deshielded peri-hydroxyl protons of hypericins in the region of 14–15 ppm in CD_3OH solutions [160]. The high-throughput analysis of 80 phytopreparations of the same plant was also efficiently performed with a near-infrared spectroscopic (NIRS) method. Hypericin, pseudohypericin, and the phloroglucine derivative hyperforine were quantified simultaneously, after subsequent chemometric calculations, in the very short analysis time of a few seconds [161]. In the case of ginseng, metabolic fingerprinting of different preparations was performed by ^{1}H-NMR spectroscopy in less than 15 minutes without any prepurification steps. Improvement in the resolution of the ^{1}H-NMR spectra was obtained by two-dimensional J-resolved NMR measurements. Multivariate data analysis (MVDA) was applied for the analysis, and principle component analysis (PCA) showed a clear discrimination among the samples. The results demonstrate that not only ginsenosides but also alanine, arginine, choline, fumaric acid, inositol, and sucrose differentiate the preparations from each other [162]. For other medicinal plants,

such as *c*, popularly employed in Mexico, MVDA applied to crude extracts from different wild plant populations successfully assesses quality and enabled a linkage of the neuropharmacological effects to given chemical profiles [163].

Thus, as shown, direct detection of given metabolites can also be performed without HPLC separation. Such approaches, however, are not applicable to many types of samples and require that specific signals can be localized within the total extract ^{1}H-NMR spectra. However, this technique represents a very interesting complement to the classical HPLC profiling approaches and to LC-NMR.

12.5 CONCLUSIONS

As shown, NMR detection in complement with other LC-hyphenated techniques, either in direct or indirect hyphenation with HPLC, is playing an increasingly important role as a strategic tool to support phytochemical investigations for the search of new bioactive lead compounds. Even if relatively insensitive compared to other analytical detectors, NMR remains the technique that will unambiguously solve the structural identification issue related to a given LC peak. Information from LC-DAD-MS is helpful and can be very precise, especially if high-resolution MS instruments are used for molecular formula assignment. This approach, in combination with chemotaxonomic considerations from cross-searching NP databases, enables the acquisition of a first dereplication step. It is, however, limited because of the lack of commercially available UV-DAD, LC-MS, or LC-MS/MS databases.

NMR used in direct or indirect hyphenation brings key complementary structural information on the order of the atoms and their stereochemical orientations for each metabolite separated by HPLC. According to the needs, as shown, NMR can be hyphenated to the LC system in different ways. On-line LC-NMR is rather simple to operate and rapidly provides important ^{1}H-NMR information on major peaks. Direct analysis of the on-flow contour plots reveals similarities found in groups of related secondary metabolites. This is interesting for a rapid chemical screening of the main compounds of an extract; furthermore, a direct estimation of the relative quantities of these products can be obtained, since NMR provides an absolute quantification of protonated species. On-flow NMR also represents an invaluable tool for the investigation of unstable compounds, since sample handling is very limited.

The operation of NMR on-line necessitates, however, some compromises due to the low sensitivity inherent to this spectroscopic method. The need for solvent suppression and the problems of chemical shift differences observed in typical reversed-phase solvent mixtures represent some major limitations. Thus, this direct hyphenation of NMR did not provide high-quality NMR data. Indeed, for in-depth structural investigations, indirect hyphenation of NMR offers numerous advantages, and alternative approaches such as LC-SPE-NMR or CapNMR should be considered. As demonstrated by the numerous applications listed, these at-line techniques are becoming more frequently used in the field of NPs.

Indeed, the complete de novo structure determination of NPs often requires access to ^{13}C information, as well as to different sets of 2D NMR spectra. These types of spectra can be provided, to some extent, by direct hyphenation of LC-NMR in the stop-flow or loop storage mode. However, acquisition in this mode still suffers from the compromises that have to be made for the solvent-suppression issues, and because of LC peak broadening during extract profiling, the elution volumes are not optimized for the highest sensitivity. SPE-NMR and CapNMR thus represent robust methods for rapid at-line identification of NPs. These methods require more sample handling and automation than do the direct hyphenation of NMR methods. However, this represents only a small percentage of the time already required for the NMR acquisition of time-consuming 2D correlation experiments. With such at-line methods, an entire set of 2D NMR experiments can be recorded with a few tenths of a microgram of compound, with only minute amounts of deuterated solvent. The SPE-NMR approach has the advantage of being fully automated in commercially available systems; it requires good optimization of the trapping parameter, but this is now well documented for many NPs. The CapNMR approach presents the advantage of a free choice of

deuterated solvents, since the samples are dried. The sensitivity of CapNMR is enhanced for sample-limited applications, and low residual solvent background signals are recorded because of the very small active volume of the flow cell. Fully automated operations are compatible with the 96-well plate format. In this case, however, solubility problems might represent an issue for some NPs, especially when 2D NMR experiments need to be recorded, since concentrations in the low millimolar range are required. Alternative methods combining the use of disposable capillarity tubes in 1 mm ID probes are also available. Other at-line approaches involving sensitive cryogenated probes for sample-limited applications are also valuable and allow the use of more dilute solutions if solubility problems are encountered; but the cost of this type of technology is considerably higher. Commercially available cryo-inserts adapted for LC-NMR flow cell also enhance the sensitivity of on-flow, stop-flow LC-NMR, or SPE-NMR approaches.

When working with crude extracts, one important task is to develop high-resolution LC methods, to avoid problems of coelution that might hamper the correct determination of a structure. In this respect, the use of modeling software to predict optimum separation conditions and the careful transfer from high-resolution analytical scale to semipreparative scale, might represent a strategic advantage.

Thus, as shown, there is no unique strategy for rapidly obtaining NMR information on NPs directly related to their corresponding LC peaks, either for dereplication or for complete on-line identification. With the state-of-the-art methods described herein, a full structural investigation can be performed in the low microgram range, while ^{1}H-NMR spectra can be recorded in the high nanogram range. This represents a considerable change in the order of magnitude, since classical methods required microgram amounts for the same type of experiments.

The impressive technological improvements of LC-NMR, and particularly of the related at-line methods, open a new field of investigation in NP chemistry where rapid chemical screening with minute amounts of extracts becomes practically feasible. Besides these important progresses in the analytical methods, it is also essential to establish a direct link between dereplication processes and biological screening via sensitive bioassays that have to be performed on-line or at-line.

Drug discovery platforms that integrate both bioassays and rational and rapid identification of NPs will thus increase the pace at which new bioactive NPs will be discovered, and the change of scale from milligrams to micrograms will extend the fields into which biodiversity can be explored.

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13 Photodiode Array (PDA) and Other Detection Methods in HPLC of Plant Metabolites

Wojciech Markowski and Monika Waksmundzka-Hajnos

CONTENTS

13.1 INTRODUCTION

13.1.1 General Characteristics of Plant Metabolites

Compounds present in plants can be divided into three groups [1,2]. The first group consists of substances occurring in all plant cells and playing a fundamental role in both metabolism and cell reproduction. Such compounds as nucleic acids, common amino acids, and saccharides belong to this group. Generally, this group of metabolites are called primary metabolites [3]. Substances used in the building of plant cell structure are present in the second group of metabolites. Substances characteristic for a limited number of specific plants constitute the third group of metabolites, which are called secondary metabolites [4,5]. Most primary metabolites manifest their biological activity within cells or organs in connection with reproduction. Secondary metabolites are also interesting because of their activity on other organisms [6,7].

Active plant components raise a lot of interest in organic chemistry because they can be used in medicine, as industrial materials, or as poisons [4]. It is claimed that about 40% of drugs used in medicine are connected with compounds derived from plants [8]. Natural compounds of plant origin play an important role in ecology as factors regulating plant interactions with each other and with microorganisms, insects, animals, and so on. In the majority of cases such compounds play the role of protective substances, antifeedants, attractants, or pheromones [6,9,10]. The presence of specific metabolites is the principle of chemotaxonomy [11]. Phytochemical inspection of plants can reveal the components that are markers in evolutionary interrelations in botany [12]. The division into primary and secondary metabolites and their most important functions are presented in Figure 13.1 [4].

13.1.2 The Chemical Character of Metabolites

The primary metabolites are of vital importance, and they are essential elements in the production of biomolecular polymers. As they belong to the classes of carbohydrates, amino acids, and fats, they include typical structural elements for these classes of compounds. Despite their wide diversity, most compounds classified as secondary metabolites belong to one of the following classes: polyketides and fatty acids, terpenoids and steroids, phenylpropanoids, alkaloids, selected amino acids, and carbohydrates. They have characteristic structural elements that result from the process of their biosynthesis in nature [13]. Examples of such groupings are acetate units (ethanoate) occurring in polyketones, isopropoenic units occurring in polyphenols, and isopropoene in terpenes and steroids.

13.1.3 Methods of Metabolite Analysis

Metabolites play an important role in organisms as mediate chains in biochemical processes. The concentration and properties of enzymes decide on the metabolite level. It means that the quantity of metabolites found in an organism is a complex function of various regulating processes that occur in cells. In this way, the metabolite level provides information about cell functions and defines the phenotype of cell or tissue in response to genetic or environmental changes. Metabolite analysis, according to *Villas-Boas et al. [14],* can be divided into:

- Target analysis connected with the identification and quantitative analysis of particular compounds
- Metabolite profiling—tracking the presence of all metabolites detectable using a selected analytical technique

Metabolite profiling may consist in the detection of intracell metabolites (metabolic fingerprinting) using a particular methodology of sampling, extraction, and analysis [15,16]. In many cases the

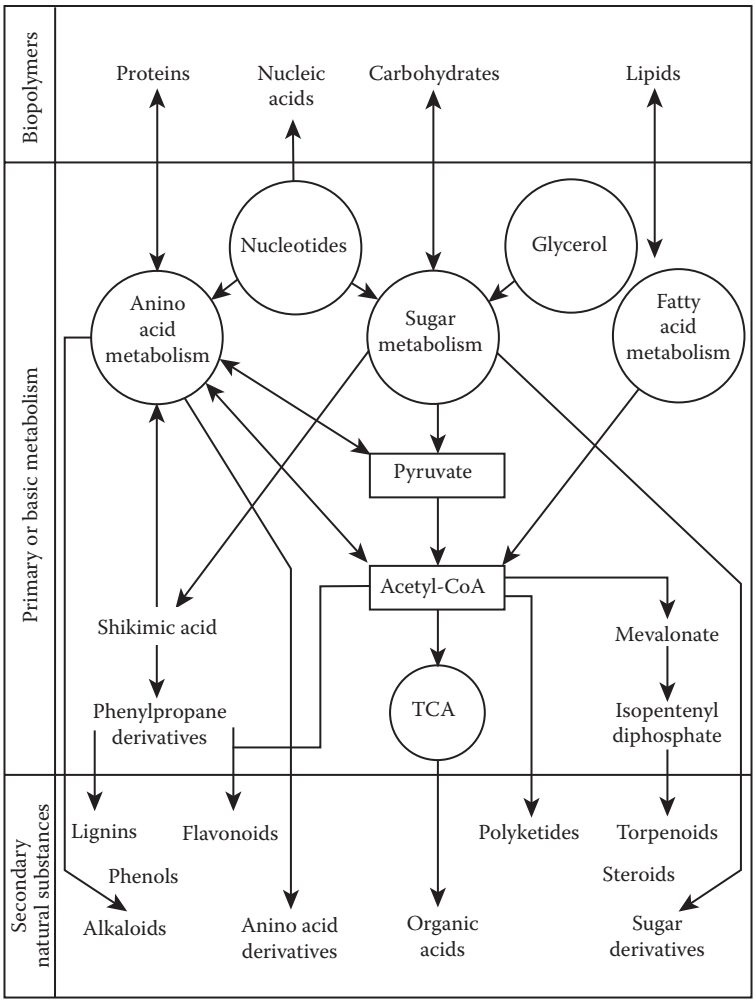

FIGURE 13.1 Relationships of biosynthetic pathways leading to secondary constituents in plants. (Reprinted from Nyiredy, S., *J. Chromatogr.*, 812, 35–51, 2004. With permission.)

analysis is limited to qualitative statements. The analysis of metabolites that are excreted outside plant tissues is defined as footprinting. Figure 13.2 illustrates the dependences within the three areas of metabolite analysis that have been mentioned [14].

The next phase in the analysis of secondary metabolites relates to structure elucidation and includes the following stages: the characteristics of the physicochemical properties of a compound, its molecular formula and carbon skeleton, the kind and position of substituents, and the stereochemical structure [17,18].

13.1.4 Separation Methods Applied in Metabolite Analysis

Recently, a lot of analytical techniques have been applied for the detection and quantitative and qualitative analysis of metabolites of natural origin and biological matrices [19–25]. Analytical techniques used for such purposes can be divided into chromatographic and other methods. In chromatographic methods, gas chromatography and liquid chromatography (LC) with various detection modes can be applied [26] (Figure 13.3). The most important detection methods applied in high performance liquid chromatography (HPLC) are ultraviolet-visible (UV/VIS), photodiode-array

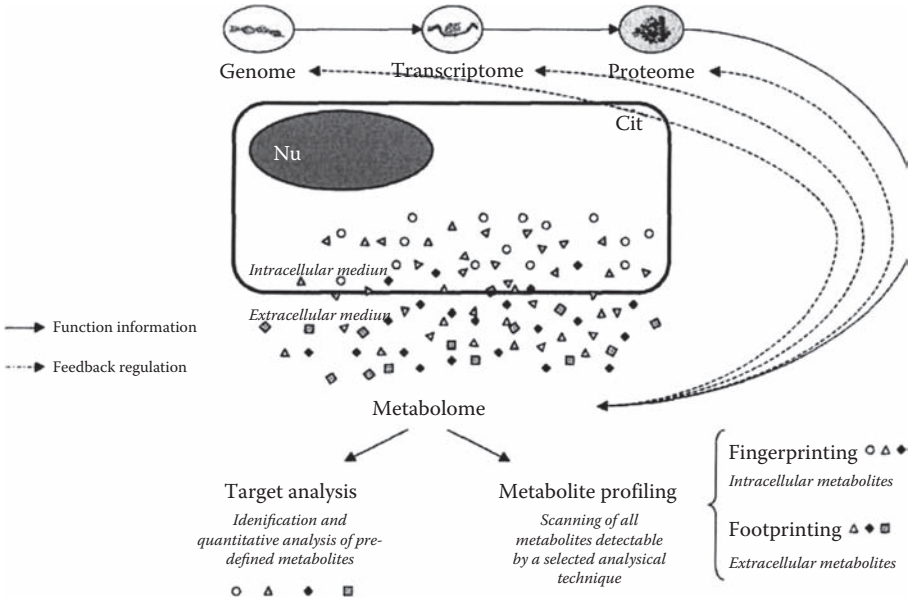

FIGURE 13.2 Metabolome analysis in the context of functional genomics. Nu, nucleus; Cit, cytoplasm. (Reprinted from Villas-Boas, S.G., Mas, S., Akesson, M., Smedsgaard, J., and Nielsen, J., *Mass Spectrom. Rev.*, 24, 613–646, 2005. With permission.)

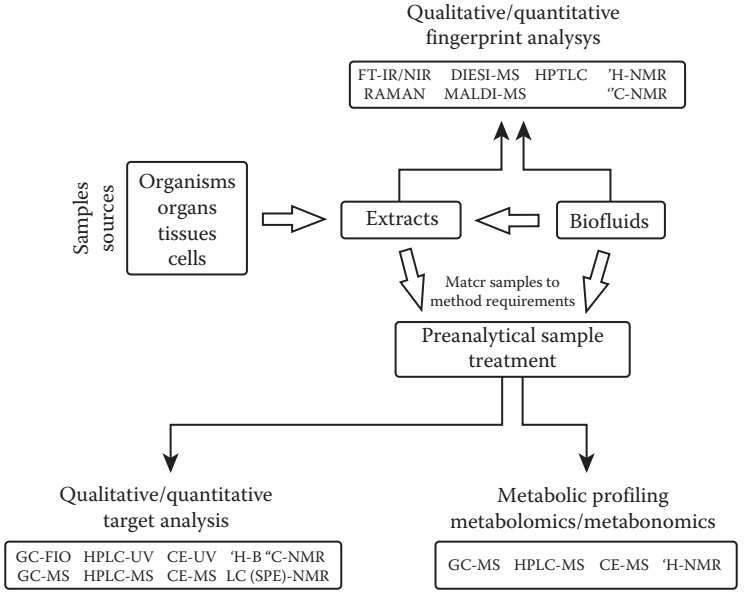

FIGURE 13.3 Flowchart summarizing preanalytical sample workup and analytical technologies utilized in metabolone analysis. (Reprinted from Seger, C. and Sturm, S., *J. Proteome Res.*, 6, 480–497, 2007. With permission.)

detectors (PDAs, DADs) [27], evaporative light scattering detectors (ELSDs) [28,29], corona charged aerosol detectors (CADs), refractive index detectors (RIDs), fluorescence detectors (FLDs) [30], and infrared (IR) [31], electrochemical [32], radiometric [33], circular dichroism (CD), and optical rotation detectors [34–37] as well as mass spectrometric (MS) and nuclear magnetic resonance (NMR) [38,39] methods. The last two methods are carried out mainly as hyphenated methods

in combination with other detection methods mentioned [40,41]. Another important method applied in metabolite analysis is capillary electrophoresis [42–45], connected with the same detection methods as already described and also immunoassay methods [46,47].

In the years since HPLC was introduced as a standard method in analytical laboratories, permanent progress has been observed in its instrumentation and computerization. The progress can be addressed to two areas: (a) the production of new sorbents allowing for a chemical character and particle size, such as particles < 5 μm, applied in UPLC, and (b) detection methods exploiting new techniques and connecting already-existing ones, such as LC-MS-NMR and automation [48–51].

The analysis of plant metabolites is not a simple task because of the huge diversity of compounds that occur in plants. Therefore, the mastering of methodology based on certain general rules is very important [52]. The use of the scheme in Figure 13.4 together with knowledge of the chromatographic process permits to one reduce the number of necessary experiments.

One of the first steps within the development method is to obtain preliminary information about the sample and formulate the aims of the analysis. Depending on the character of the sample and its complexity, it may be necessary to determine the use of special sample-preparation procedures [53,54]. The next step is the choice of detector or detection methods. In this case, either a universal detector that enables the statement of presence of all sample components (RID, ELSD, CAD) or a selective detector enabling the statement of presence of most sample components should be chosen.

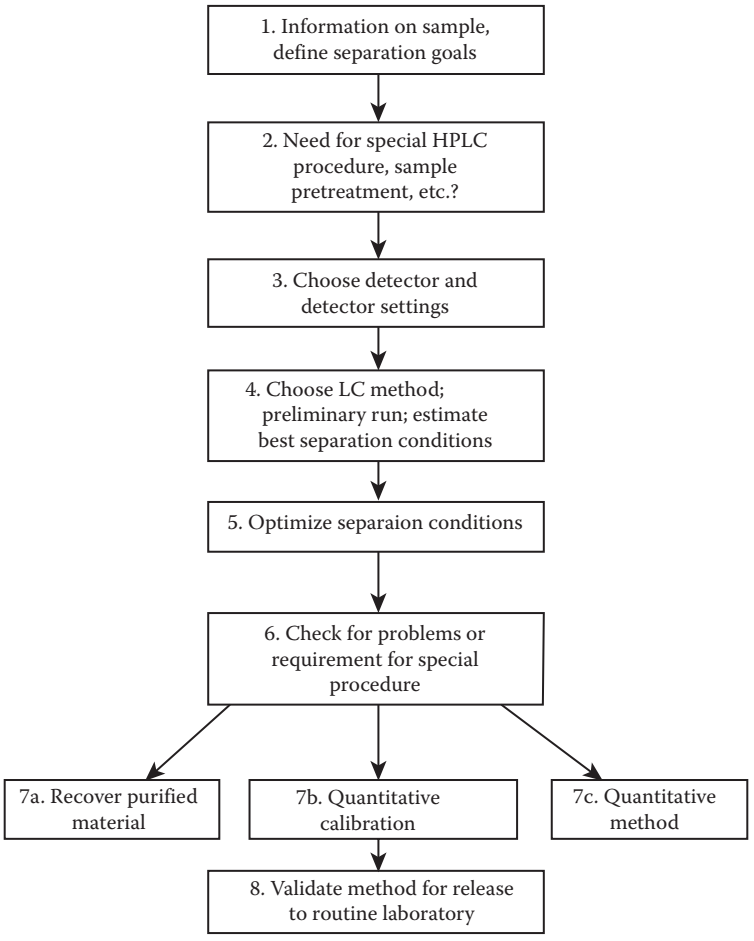

FIGURE 13.4 Steps in HPLC method development. (Reprinted from Snyder, L.R., Kirkland, J.J., and Glajch, J.L., *Practical HPLC Method Development*, Wiley, New York, 1997. With permission.)

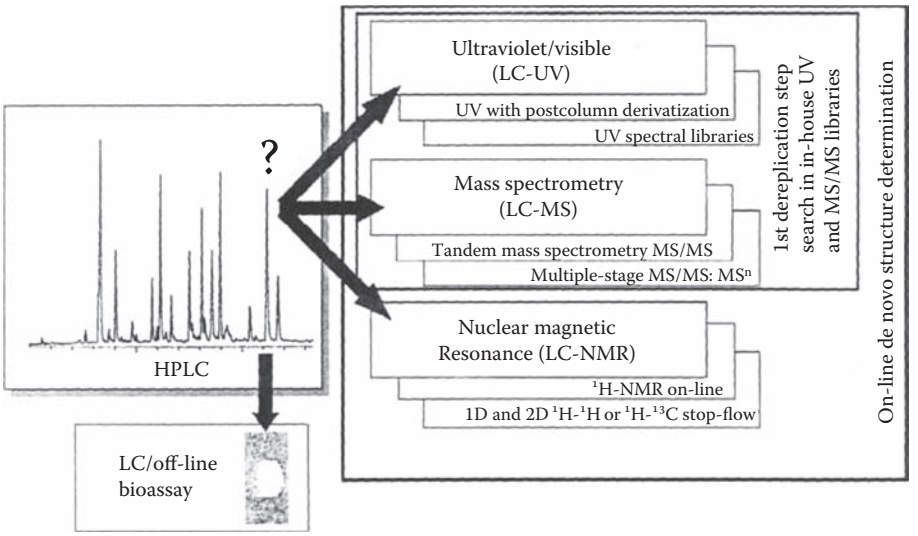

FIGURE 13.5 Type of information that can be obtained on a given liquid chromatography (LC) peak with different LC-hyphenated techniques available. In this approach LC–nuclear magnetic resonance (NMR) can be regarded as an efficient addition to LC–UV–mass spectrometry (MS) for de novo structure identification of a natural product on-line. (Reprinted from Wolfender, J.L., Nadjoko, K., and Hostettmann, K. *J. Chromatogr. A*, 1000, 437–455, 2003. With permission.)

In the latter case, the best choice is a DAD detector applicable for the majority of secondary metabolites. Gathering of UV/VIS spectra for sample components provides valuable information that can be applied in further methods of development. In other cases, when the application of the UV/VIS spectrum is not possible or ineffective, the use of other detectors such as fluorescent or electrochemical ones should be taken into account. Since samples of plant origin are complex, the use of single detection methods is rarely applied. Several techniques are often connected, mainly combinations of LC-UV, LC-DAD, LC-MS, and LC-NMR such as LC-DAD-MS, LC-DAD-MS/MS, or LC-DAD-NMR. An example of the connection of liquid chromatography with other techniques as well as the obtained information is shown in Figure 13.5.

13.2 FUNDAMENTALS OF DETECTION IN LIQUID CHROMATOGRAPHY

The detector is an instrument that consists of a sensor and electronics [55,56]. The general task of the detector in HPLC is to track the presence of sample components in the medium, leaving a column. In the detector, the formation of an electric signal that is proportional to the concentration or quantity of the component in the eluent leaving the column should occur. The signal produced in the detector is further processed and registered. The detector has to work with precision, high sensitivity, and stability. One of the most important tasks of the detector, especially in the wake of the progress in the field of column and sorbent production and their performance, is to reduce the loss in the separation/resolution of mixture achieved in the chromatographic system. Therefore, a solution concerning the construction and referring both to connecting pipes and the detector itself should be applied in order to obtain a minimal diffusion of zone leaving a column [55,56].

In turn, the progress in obtained efficiency of chromatographic columns required new solutions in the construction and functioning of detectors as well as in the electronics connected with the detection process (detector time constant, the use of optic fibers in electronics, programmed change of diaphragm size). This interdependence still exists and results in more modern solutions that appear in detectors and are available on market, such as the detector cell set for short columns 2–5 cm long filled with 2–3 μm particles of adsorbent. Exchangeable detection cells that are in

equipment of modern liquid chromatographs used in semi-micro, microbore, semi-preparative, or LC-MS processes are another example [55–63].

13.2.1 Classification of Detectors

In chromatographic literature there is an agreement concerning the criteria used in the classification of detectors, but there is no uniform view on the significance of particular classifications [55–64]. Practical consequences that result from the accepted division seem to be the most important aspect of detector classification. If several physicochemical properties of the eluent flowing from the column are taken into account and compared with the same properties of the eluent containing sample components (eluate), such detectors are classified as bulk property detectors. An example of such a detector is the RID, which works on the basis of measuring the light refraction coefficient. The signal is proportional to the eluate's refraction coefficient. Such a detector identifies all compounds.

The detectors in which the created signal depends on the presence of a component having specific properties in the eluate flowing from the column are another class of detectors called solute property detectors. In an ideal case, the eluent is assumed to be inactive and does not reveal specific properties typical for the substance. An example of instruments in this class are UV detectors. The signal is formed when a substance that absorbs light in UV range is present in the eluent. Obviously, in the case of detectors such as a UV detector, the properties of eluent (absorbance in UV range) should be taken into account. As was already mentioned, practical aspects resulting from such a classification are very important. In the case of the first class of detectors, such devices do not indicate practical application in the analysis by gradient elution. In the case of the second group of detectors, their transparency in UV range should be taken into consideration while selecting the components of the eluent.

Assuming a practical point of view, there is also a classification of detectors into those that detect all sample components, a particular group of compounds, or only one compound. The first group of detectors is called universal, the second selective, and the last specific [55–64]. Another classification is connected with the proportionality of signal to the quantity of component in a measuring cell of the detector. If the signal is proportional to the concentration of solute, the detector is called a concentration detector. If the signal is proportional to the mass stream, the detector is called a mass detector. A practical consequence of such a division is both the influence of eluent flow velocity on the surface and the height of concentration profiles registered by the detector [59]. The third aspect of detector classification is the dimensionality of the obtained information. Thus, in the simplest UV/VIS detector, the information is obtained in the following form: $A = f(t)$ (where A-absorbance; f-function of; t-time) for the chosen wavelength λ. However, the use of DAD allows the obtaining of simultaneous information in form $A = f(t, \lambda)$, which in connection with chemometric methods allows one to determine the number of components and the spectra of each component in the mixture [63].

13.2.2 The Ideal Detector and Comparison of Detectors

In frequent cases, the application of the procedures elaborated in various research groups, or the use of the same detector type coming from various producers, may result in a failure since the parameters that are typical for one detector might differ significantly from the detector parameters that are applied in the adopted procedure. In order to avoid such types of situations, The International Union of Pure and Applied Chemistry (IUPAC) has defined the way in which detectors should be characterized to compare them with one another. Such characteristics include the following data: sensitivity, that is, the minimal detectability, often referred to as the limit of detection (LOD), as well as the minimal concentration used in quantitative determinations, called the limit of quantification (LOQ) [55–63]; linearity; dynamic linearity range; noise level; sensitivity with respect to temperature; pressure; and flow (Figure 13.6).

One of the most important detector characteristics is its linearity (D_{LR} – $g \cdot mL^{-1}$). Linearity determines the precision of quantitative measurements by the HPLC method. According to Scott

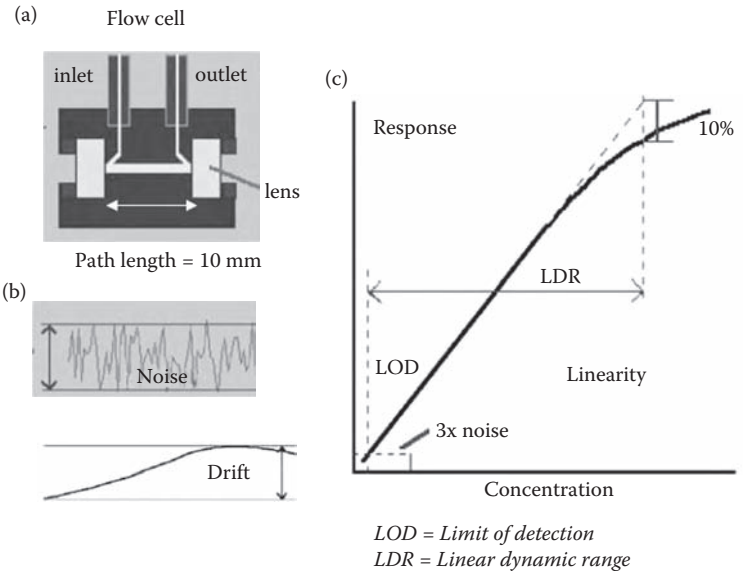

FIGURE 13.6 (a) Schematic diagram of a flow cell and (b) baseline chromag noise (magnified) and drift. (c) Chart of UV response with concentration of the analyte injected. Linearity range is from limit of detection (LOD) to the point deviating 10% from the linear response. (Reprinted from Dong, M., *Modern HPLC for Practicing Scientist*, John Wiley & Sons, Inc., Hoboken, New Jersey, 2006. With permission.)

and Fowlis [55], the dependence of the signal on the concentration of solute in the eluate can be described by the following equation:

$$R_C = k \cdot C^{\psi}, \tag{13.1}$$

where R_C (specific units of measurement/g) stands for the response of the detector on the concentration C of solute in the eluate, ψ is the response index, and k is constant.

In the case of an ideal detector, the response index ψ is equal to 1, and for almost linear detectors it is assumed that the response index should range <0.98–1.02>. The response index can be applied to introduce appropriate corrections in quantitative analysis when its values are out of the optimal range [65]. Outside of signal-to-concentration proportionality range, there exists the area in which the detector reacts to the concentration stimulus without retaining proportionality, although the logarithmic or exponential manner is still retained. This area is called the dynamic range, D_R – g·mL^{-1}, as opposed to the prevoiusly discussed linear dynamic range. Another important parameter that characterizes the functioning of the detector is the level of noise and the drift of the baseline. A practical conclusion that results from the considerations upon the noise and the baseline drift is a rule that says that sensitivity should not be set over 2% FSD (full-scale deflection) for a computer-simulating chromatogram [55].

Detector sensitivity is often defined as the minimal detected concentration and is registered as

$$X_D = \frac{R_C}{N_D} \geq 2, \tag{13.2}$$

where X_D (g·mL^{-1}) is the sensitivity or minimal detected concentration, R_C is the response of the detector, and N_D is the noise level of the detector, most often expressed in specific units (e.g., for UV in absorbance units). Giving the sensitivity of the detector is useless if it is not given together with the substance for which it has been determined. Parameters such as pressure, flow, or temperature are crucial from the perspective of sensitivity, therefore, and the influence should be specified by the producer. The comparison of the most popular detectors used in HPLC is presented in Table 13.1.

TABLE 13.1
Common HPLC Detectors and Attributes

Detector	Concentration/Mass Flow Rate. Bulk Property/ Solute Property	Analyte/Attributes	Sensitivity	Compatibility with Gradient/ Destructivity/Linear Range
UV/VIS	Concentration/solute	Specific: compounds with UV chromophores	ng-pg	Gradient/nondestructive/$10^3 \div 10^4$
PDA/DAD	Concentration/solute	Specific: same as UV/VIS detectors, also provides UV spectra	ng-pg	Gradient/nondestructive/$10^3 \div 10^4$
FLD	Concentration/solute	Very specific: Compound with native fluorescence or with fluorescent tag	fg-pg	Gradient/nondestructive/$10^3 \div 10^4$
RID	Concentration/bulk	Universal	0.1–10 µg	Not compatible with gradient/ nondestructive/10^3
ELSD	Mass flow/solute	Universal: nonvolatile or semi-volatile compounds	10 ng	Gradient/destructive
CAD	Mass flow/solute	Universal: use nebulizer technology like ELSD and detection of charges induced by a high-voltage corona wire	Low ng	Gradient/nondestructive
CLND (chemiluminescence nitrogen)	Concentration/solute	Specific to N-containing compounds based on pyro-chemiluminescence	<0.1 ng of nitrogen	Not compatible with gradient/destructive, not compatible with any N-containing mobile-phase component.
Electrochemical	Mass flow/solute	Very specific: electro-active compounds (Redox)	pg	Gradient/destructive
Conductivity	Concentration/bulk	Specific to anions and cations, organic acids, surfactants	ng or ppm-ppb	nondestructive
Radioactivity	Mass flow/solute	Specific, radioactive-labeled compounds	Low level	10^3
MS	Mass flow/solute	Universal and specific, structural identification; very sensitive and specific	ng-pg and pg-fg	Gradient/destructive/10^7
NMR		Universal, for structure elucidation and confirmation	mg-ng	Gradient/nondestructive
CD	Concentration/solute	Specific to chirality of compounds		Gradient/nondestructive

Note: UV/VIS: absorbance; PDA/DAD: photo diode array; RID: refractive index; ELSD: evaporative light scattering; CAD: corona-charged aerosol; MS: mass spectrometry; NMR: nuclear magnetic resonance; CD: circular dichroism.

13.3 SPECTROPHOTOMETRIC DETECTORS

13.3.1 UV and DAD Detectors: Construction and Operating Principles

Spectrophotometric detectors function based on the interaction of electromagnetic radiation with matter in the range from near UV (190 nm) to near infrared (1100 nm). A fundamental dependence that measurements using such detectors are based on is the Beer–Lambert law, which combines the absorbance with the concentration, path length, and molar absorptivity. Spectrophotometric methods are very useful for quantitative analysis, although little information concerning the structure elucidation of analytes is obtained.

Figure 13.7 presents a basic block scheme of the UV/VIS and PDA detectors. Using spectrophotometric detectors, the measurements can be performed by one wavelength, most often at 254 nm or by other wavelengths chosen from the range of the UV/VIS spectrum. Recently, the photodiode detector (linear photodiode detector [LPAD] or diode array detector [DAD]) has been the most frequently used spectrophotometric detector. This type of detector was introduced onto the market by Hewlett-Packard in 1982 [63]. Spectrophotometric detectors with the stop-flow option for the absorption measurement in the entire range of the spectrum were the precursors to the detectors mentioned previously. In the DAD detector, the changes of the wavelength are programmed during the time of analysis, which is possible thanks to the application of photodiode technology and modern software with adequate computer equipment.

The advantages of the DAD detector can be explained using the following example: If a monochromatic detector is used ($\lambda = 254$ nm), the process of gaining information about the components of the sample finishes together with the end of an analysis. If the sample contains a component that does not absorb at the chosen wavelength, the analysis should be repeated using a different wavelength. The use of DAD allows one to obtain the information and register it in the form of $A = f(\lambda, t)$ at a given moment. Having finished the analysis, there is a possibility to return

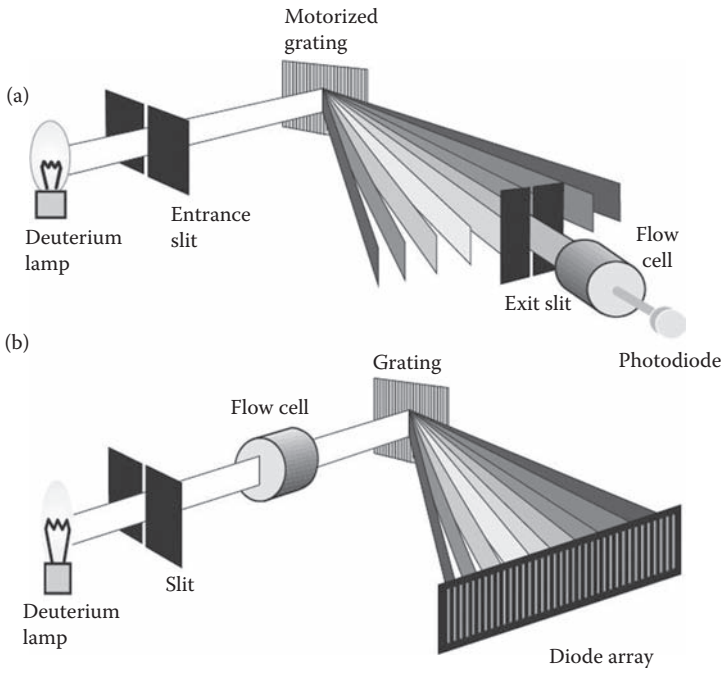

FIGURE 13.7 A schematic of (a) a UV/VIS absorbance detector and (b) a photodiode array (PDA) detector. (Reprinted from Dong, M., *Modern HPLC for Practicing Scientist*, John Wiley & Sons, Inc., Hoboken, New Jersey, 2006. With permission.)

to the registered information (file) connected with the absorption measurement at a different wavelength. The use of chemometric methods while elaborating the DAD data brings additional benefits [67].

The DAD consists of a series of photodiodes. Photodiodes are placed in a way that allows the rays of a determined wavelength obtained by the dispersion of light on holographic diffraction reticle to fall on each of them. For example, a set of diodes consists of 211 units, all 50 mm wide. Each unit is responsible for the absorption measurement in a very narrow range of the wavelength. Diodes are connected with condensers and transistors. At the start of the measurement cycle, the moment when the light falls on the diode, an electric current is produced, which causes partial discharge of the condenser. The electric current, which is indispensable to recharge the condenser, is proportional to the light intensity that caused the condenser discharge. An absorption spectrum can be obtained measuring the changes in light intensity in the UV/VIS range [68–74].

13.3.2 The Application of the DAD

13.3.2.1 The Detection of Sample Components Absorbing in UV/VIS Range

While elaborating a chromatographic method, information about the absorption of the investigated components in the UV/VIS range should be collected. With this in mind, both an appropriate wavelength ranging 190–600 nm while measuring the absorption, as well as a high value of the spectral width should be chosen. With conventional detectors, the injection of the sample should be done repeatedly, changing the wavelength each time. DAD enables the problem to be solved at one run.

As soon as it has been checked which components absorb in the UV/VIS range, the next step consists in the determination of the wavelength at which particular peaks indicate the maximum absorption. The wavelength that corresponds with the maximum can be searched by several methods: (a) the automatic choice of the wavelength by setting a suitable option in the method applied (software accompanying the instrument) or (b) the reference to the color izoabsorbance map, which presents the projection of $A = f(t, \lambda)$ on the surface. Choosing option b, a chromatogram that corresponds with the position of a horizontal cursor and the absorption spectrum corresponding with the position of a vertical cursor is projected on the screen. Operating with cursors respectively, it is easy to choose the wavelength at which the searched components will be detected with significant sensitivity [75].

13.3.2.2 Quantitative Analysis of Chromatographically Unseparated Components

When two substances are chromatographically unseparated, it is not possible to determine them quantitatively by the use of a monochromatic detector [76]. Using the detector with a double wavelength does not allow the separation of such substances as well because the UV spectra overlap. The problem can be solved using the method of so-called peak suppression, which is presented in Figure 13.8. In the case of DAD, quantitative determination is possible, even if peaks are chromatographically unseparated and the spectra overlap in all wavelength range. The method consists in the absorption measurement at the chosen wavelength and the so-called reference wavelength [64].

13.3.2.3 Purity Peak Analysis

The crucial problem in the elaboration of the chromatographic method and the quality control is to check whether the peaks visible on the chromatogram correspond with pure compounds or whether they contain impurities. Such an estimation is impossible on the basis of a single chromatogram. The purity degree can be verified using a DAD if the spectra of a compound and its impurities differ from each other. There are several methods that check the purity of peaks. The most popular method consists in getting a few spectra in the time of elution. The comparison can be later performed by the normalization and superimposition of spectra. Another option consists in choosing the automatic peak purity checking by the software connected with the instrument. If the spectra overlap in all the wavelength ranges, it means that a peak corresponds to a pure compound. A minimal number of points chosen on the chromatographic peak to check the purity is 3. These should be

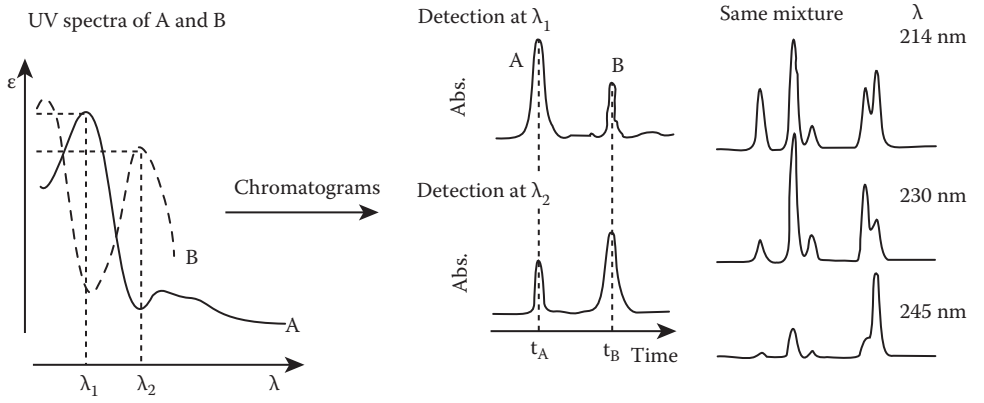

FIGURE 13.8 Chromatogram of sample containing two compounds, A and B, for which the UV spectra are different. According to the choice of detection wavelength, the chromatogram will not have the same aspect. On the right the chromatogram represents a mixture of several pesticides recorded at three different wavelengths, which illustrates this phenomenon. (Reprinted from Rouessac, F. and Rouersac, A., *Chemical Analysis: Modern*, John Wiley & Sons, Ltd. Chichester, England, 2007. With permission.)

points corresponding to the rising part, the maximum, and the falling/descending part of the peak. The spectra chosen for these points are further normalized and compared. The comparison and estimation can be visual or based on the calculation of the matching factor [63].

13.3.2.4 The Identification of Peaks on the Chromatogram

In LC, it is the retention time or relative retention that is used for identification. The additional method of peak identification is the use of the UV/VIS spectrum. For this purpose, the standard of a component or spectra library should be available. Using a DAD for the identification, the spectra obtained in peak maximum should be taken into account and compared with the spectra available in the library. Having compared the above parameters, the matching factor is calculated, using the software [63]. Other tasks that can be performed using DAD are checking the coelution of components and the elution sequence if gradient elution is applied.

13.3.2.5 Trends in PDA Detectors

Modern DADs have sensitivity performance on the benchmark level of 1×10^{-5} AU. Further sensitivity enhancement has stemmed from an innovative design of the flow cell using light-pipe (fiber-optics) technology, which enables the extension of the path length without increasing the noise or chromatographic dispersion. By coating the light-pipe with reflective polymer to allow total internal deflection, very small flow cells can be constructed with a long path length (e.g., 50 mm) with excellent dispersive characteristics. Another important innovation that has been introduced recently is the application of programmable slit design, which allows the user to select for either high detector sensitivity (wider slit) or high spectral resolution (narower slit) [57].

13.4 REFRACTOMETRIC DETECTORS

The work of refractometric detectors consists in the measurement of the light refraction coefficient. The possibility of the application of such detectors in routine analysis of compounds that do not exhibit absorption in the UV range is their main advantage. Therefore, they are widely used in the analysis of sugars, lipids, and polymers [78]. Moreover, refractometric detectors can be successfully applied in gel-permeation chromatography (GPC) and size-exclusion chromatography (SEC) [79]. Keeping the temperature of the optical unit constant is a significant factor in the work of such a detector.

13.5 LIGHT SCATTERING DETECTORS

13.5.1 ELSD

The ELSD is the second type of detector used in HPLC that can be classified into the group of universal detectors. A particular advantage of such a detector is its possibility to be used in isocratic and gradient analysis. The condition for using this type of detector is the volatility of the component, which should be lower than the volatility of the mobile phase, as well as the possibility of using the detector in the case of thermally unstable compounds [69,70,80]. The components should not exhibit absorption in the UV range, fluorescence, or susceptibility to ionization. The work of such detectors consists in the transformation of solution eluted from the column into fine drops forming a colloid in air, which is next transferred across a formed spiral in which it is enriched with gas and goes to a measuring cell in which the light is dispersed proportionally to the concentration of any compound. As was already mentioned, the only condition needed to allow detection of compound molecules is that their volatility be lower than that of the components of the mobile phase. Getting the lowest temperature, which increases sensitivity and decreases the level of noise, is crucial in nebulization process. Such detectors can be used with typical analytical columns of older generation, as well as in connection with rapid resolution liquid chromatography (RRLC).

13.5.2 Corona Charged Aerosol Detector (CAD)

This type of detector was introduced onto the market in 2004 by ESA. It belongs to the universal group of detectors and functions in a way similar to the ELSD. The work of the detector is based on the nebulizer and the detection of charges that arise under the influence of high voltage produced by corona wire. The device enables the detection of samples whose concentration is at the nanogram level. The parameters of the CAD are significantly better than those of the ELSD [81].

13.6 FLDs

The idea applied in such detectors consists in the irradiation of the sample with an electromagnetic stream of a determined wavelength. The next step is the measurement of the radiation emitted by the sample as a result of fluoresecence. The latest technological solutions enable the collection of a full set of wavelengths responsible for both the excitation and wavelength of the fluoresecent radiation for all the components of the sample without the interception of chromatography [82–85].

13.7 CHEMILUMINESCENCE NITROGEN DETECTOR (CLND)

The CLND is designed to detect molecules containing nitrogen. Its work is based on the reaction of nitrogen oxidation to nitrogen oxide and further the reaction of the oxide with ozone, which causes emission of light of a determined wavelength. The detector is highly specific and sensitive to nitrogen-containing compounds (< 0.1 ng nitrogen). The detector signal is proportional to nitrogen molarity in an examined compound. Regarding the application, the CLND enables the detection of drug impurities and decomposition products contained in pharmaceuticals. It is not possibile to use the detector with eluents containing nitrogen solvents [86].

13.8 ELECTROCHEMICAL DETECTORS (ECDs)

13.8.1 Amperometric and Culometric

Electrochemical detectors are known for their high selectivity and sensitivity. The main principle of their work consists in the measurement of current, which is produced between the electrodes in

the flow cell. When considering the use of ECD detectors special attention should be paid to the determination of catecholamines at the pikogram level as well as compounds with amino-, diazo, and nitro functional groups. There are several types of ECDs, such as amperometric or culometric detectors [87,88].

13.8.2 Conductivity Detector

The conductivity detector is applicable to the analysis of ions and enables their determination on the ppm-ppb level. The ions of organic acids are mainly analyzed in this way [89,90].

13.9 RADIOMETRIC DETECTOR

This type of detector is highly specific, and its function is confined to radioactive compounds, indicating extreme sensitivity. The measurement consists in determining the radioactivity of the eluent flowing across the detector cell. The detector reacts to the presence of C-14 or tritium-labeled compounds and is mainly used in the investigation of degradation processes, metabolites, and toxicology [56–59].

13.10 POSTCOLUMN REACTION TECHNIQUES

In the case of analytes that do not contain any chromophoric groups, there is another detection method consisting in the transfer of the examined analytes into derivatives containing chromophores. In this way, both the detection and its increased sensitivity are possible [91–93].

13.11 INFRARED DETECTION

Getting IR spectra provides information not only about functional groups (especially OH, CO) but also about their interactions. If, for example, a hydroxyl group takes part in H-bonding, there is a possibility to obtain the correlation between the length of H-bonding and the absorption indicated in the IR spectrum. The distinction between axial and equatorial epimery is possible by absorption measurement in the region of ca. 1000 cm^{-1} for the carbon-oxygen single bond stretching [57–59].

13.12 HYPHENATED AND SPECIALIZED SYSTEMS

13.12.1 LC-MS, LC-MS/MS

Chromatographic methods that adopt a single detection form are gradually losing their importance and are often replaced by hyphenated techniques, which offer several methods of detection. Obviously, MS connected with LC and the still widely used gas chromatography takes the lead. There are techniques offering very high sensitivity. The problem that is found in LC-MS is connected with the ionization of analytes in the stream of liquid and further transport to the other parts of MS. Nowadays, a wide range of ionization methods and instruments are used for this purpose: quadrupole, ion trap, triple quadrupole, time-of-flight (TOF), and Fourier transform MS (FTMS). In most MS instruments the mass resolution is 1 mass unit, whereas the maximum mass of the analyzed compounds can be about 2000. In the case of TOF and FTMS applicaction, the mass resolution increases to 1–10 ppm. The molecular mass of substances that can be analyzed by this method also increases. The use of LC-MS/MS, which takes advantage of triple four-polar analyser, causes an increase of sensitivity and specificity, which enables trace analysis of the complex matrices. In this method, basic ions are fragmented into smaller ones,

and on the basis of the analysis of daughter ions, the structure is determined and quantitative analysis is performed [57,66].

13.12.2 LC-NMR

Not only the LC-MS hyphenated technique is possibile for metabolite analysis. The connection of LC and NMR is becoming more and more popular. The use of NMR in connection with LC is now possible owing to the progress in the construction of NMR instruments. In the first stage of the development, the NMR method required pure samples in milligram quantities, and measurements were performed by a stop-flow method. Now, it is possibile to perform LC-NMR analysis without stop-flow, and the quantity of the analyte required in the analysis was reduced to micrograms. The research on LC-NMR aims at the miniaturization, noise reduction, and so on, thus making LC-DAD-MS or NMR methods powerful investigative instruments [94,95]. Practical application of HPLC hyphenated with MS/MS and NMR and circular dichroism (CD) to the complex analysis of biological material of terrestric or marine origin is presented on Figure 13.9.

13.12.3 Other Hyphenated Techniques

LC with infrared spectroscopy, helpful in the identification of analytes, as well as LC coupled with spectroscopy such as atom absorption (AA) or inductively coupled plasma (ICP) for study of method speciation in samples, belong to this group [96].

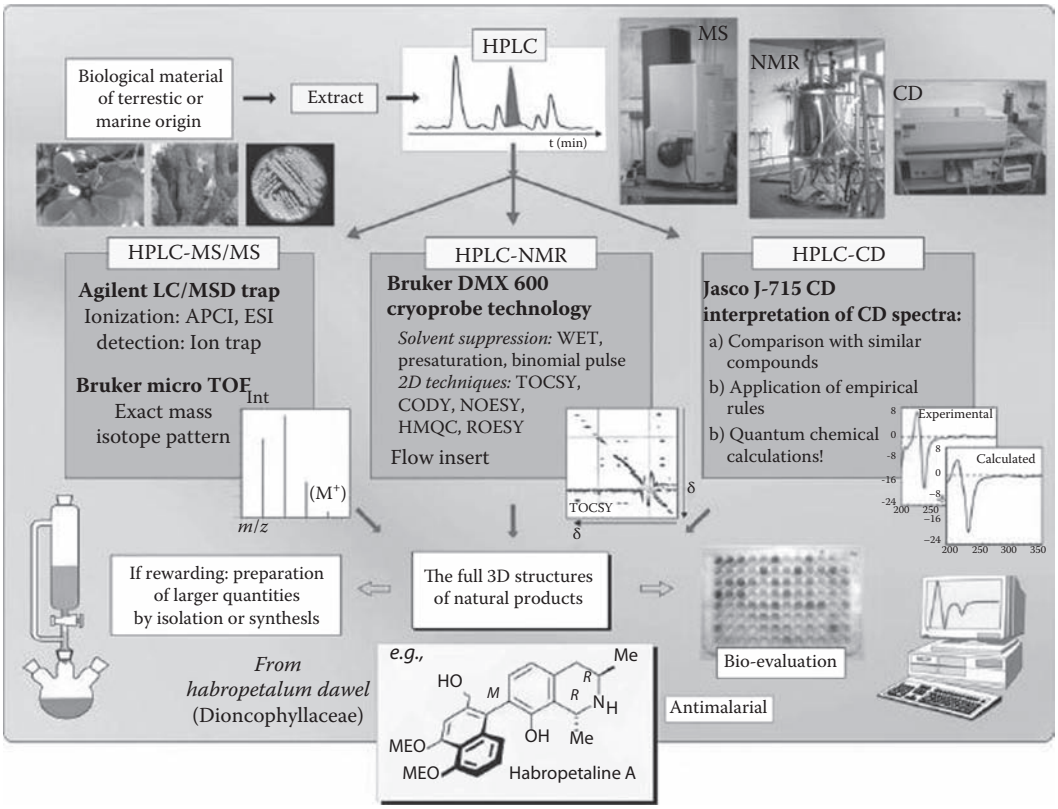

FIGURE 13.9 The HPLC method hyphenated with tandem mass spectrometry (MS/MS), nuclear magnetic resonance (NMR), and circular dichroism (CD) in analysis of biological material of terrestrial or marine origin. (Reprinted from www.organile.chemie.umi-wuersbary.de. With permission.)

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14 Quantitative Analysis—Method Validation—Quality Control

Pierre Masson

CONTENTS

14.1 INTRODUCTION

Chromatographic techniques are useful analytical methods for separation, identification, and quantitative determination of a wide variety of compounds from a wide variety of matrices. Many components of interest have been identified in phytochemical analysis (proteins, carbohydrates, lipids, alkaloids, phenolics, terpenoids, and so on), and their quantification with high performance liquid chromatography (HPLC) always follows the same rules [1]. Chromatography is primarily a comparative analytical technique. The values generated by the detector are not directly usable and must be changed in concentrations. Thus, chromatography involves a calibration that is the key operation in a chemical measurement process.

The other important operation to ensure quality of measurements is method validation. A laboratory should demonstrate that its analytical method is adapted to the targeted application. Method validation describes performances and limitations of the analytical system and identifies the parameters that may change these characteristics. Finally, the objective of an analytical procedure is its routine use. The laboratory must be able to maintain performance of the validated method in all circumstances. Consequently, an adapted quality control system is essential to ensure good traceability and comparability of data.

14.2 ANALYTICAL QUANTIFICATION IN HPLC

The detectors used in HPLC measure a signal related to the amount of analyte in the solution injected. One obtains a peak whose surface is related to its quantity by the relation:

$$m = KA,$$

where m is the mass of substance crossing the detector, A is the corresponding peak area, and K is the response coefficient. Quantitative analysis is thus reduced to the measurement of A and K. The determination of peak area can be carried out using one electronic integrator. The coefficient of response is measured from the peak surfaces corresponding to a series of standards characterized by a known value of the substance amount (or concentration). This set of operations that determines the functional relationship between the instrumental response and the amount (concentration) of analyte is termed calibration [2].

However, using HPLC, there is no universally applicable calibration procedure that can be used in all cases. Various methodologies of calibration can be applied in order to minimize errors in quantification. The calibration standards can be measured apart from the sample (external methodology) or together with the sample (internal methodology).

14.2.1 Operating Precautions

For a multianalyte analytical method as encountered with separation techniques, the calibration standards are mixtures containing the different analytes of interest in adequate amounts. Standard mixtures must be prepared from substances (single or mixed) characterized for chemical purity in the working solvent. The solvent must ensure the total solubilization of the substances. The concentration and the stability of each new standard solution must be checked with independent standards. Finally, the solutions should not give rise to degradation reactions with stationary or mobile phases.

The detector must be suitable for the operating conditions and the nature of mobile phase. Its sensitivity must be compatible with the concentrations of standard solutions. A great number of detectors based on different principles are marketed (Table 14.1). To be exploited quantitatively, obtained responses must be measurable and repeatable. To ensure that the measurement system remains in a calibration state, some periodic controls are required (possibility of drifts in the calibration factors).

TABLE 14.1
List of Detectors Generally Used in HPLC

Detector	Principle
UV/visible spectrophotometry	Absorption of radiation; the response follows the law of Beer-Lambert
Spectrofluorimetry	Absorption of radiation by substances that then emit radiation at a higher wavelength
Electrochemistry	Electrolysis of substances and intensity measurement
Conductivity	Conductance measurement
Evaporating light scattering	Diffusion of radiation by an aerosol formed by analytes after evaporation of the mobile phase
Polarimetry	Chiral molecules detection
Radioactivity	Measurement of radioactivity in spiked fraction of molecule marked with radioactive isotope
Mass spectrometry	Action of magnetic and/or electric field on a charged particle; separation and analysis of ions according their mass/charge ratio
Nuclear magnetic resonance	Interaction between a magnetic field and atomic nucleus from molecule
Infrared spectroscopy	Modification of vibrational and rotational energies of a molecule
Raman spectroscopy	Spectrometric diffusion from vibrational state of a molecule
Atomic emission	Emission of radiation from an element
Refractive index	Change in the refraction index
Viscometry	Viscosity measurement

14.2.2 External Calibration

The external calibration is based on the measurement of a set of calibration standards, including a blank, carried out successively under invariant chromatographic conditions. Then, applying a statistical regression method to the data, a calibration function is obtained for each analyte. The easiest way to describe the measurement function of an analyte is:

$$S = S_o + S_{analyte} = S_o + KC.$$

The analytical signal S depends on two components. S_o, denoted as the blank signal, is independent of the quantity of analyte and is produced by three different terms:

i. The instrumental background obtained in the absence of any analyte
ii. The signal arising from contamination from the reagents or glassware
iii. The signal in the analyte peak region of interest due to interfering species

Ideally, the analytes pass through the measurement system separated from the other components of the sample, and the blank signal should be zero. The signal ($S_{analyte}$) is generated by the analyte. $S_{analyte}$ is expressed as the product of the sensitivity of the measurement system, K, not necessarily constant, and the amount of analyte (C). The linear function is the most frequently observed in analytical science. The linearity of an analytical method is its ability to obtain results that are directly proportional to the concentrations (amount) of the analyte in the sample. In some instances, HPLC data systems offer a number of mathematical solutions to the nonlinearity of the calibration curve (polynomial, logarithmic, or exponential functions). The correlation coefficient (r) is calculated by the least squares regression and provides some information about how well the computed line fits the calibration data points.

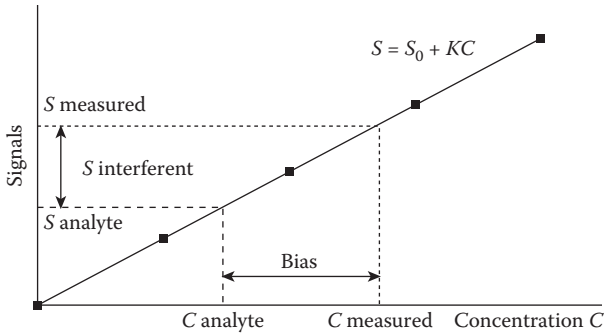

FIGURE 14.1 The matrix effect. The measured signal corresponds to the summation of the signal of the analyte and the signal of the interferent. The calculated concentration then presents an analytical bias.

To carry out the quantification of an analyte in a sample, the corresponding output analytical signal (S) is measured, and a numerical value of the input analytical concentration (C) is calculated from the inverse calibration function. Many representative examples of this procedure in phytochemical analysis are available: analysis of lipids [3], carbohydrates [4], terpenes [5], flavonoids [6], alkaloids [7], or amino acids [8] in plant samples.

It is admitted that analytes in samples and standards have identical behavior in the measurement system and that the sample matrix has no effect on the calibration factor value. The main limitation of external calibration derives from this assumption. The matrix of a sample corresponds to all major and minor components that constitute "the environment" of the substance to be measured. The matrix can disturb the instrumental signal, and then the analyte measurement will be biased (Figure 14.1). Matrix effects are generated by various physical and chemical processes. They can be constant and independent of the analyte concentration or variable and proportional to the analyte concentration. A matrix effect may be demonstrated by comparing the pure analyte response in a solvent solution with that obtained from the same amount of analyte in the presence of the sample extract. Matrix effects are often variable, unpredictable, and difficult to eliminate.

14.2.3 The Number of Standards

The multistandard calibration is the most commonly employed calibration methodology. In general, the number of standards varies from one to six according to the calibration curve and the applications. A significant source of bias in analytical measurements can be caused by an inadequate choice of the mathematical model to describe the calibration curve. When the linearity of the calibration function has been well established during the validation step, a routine calibration can be obtained from only two-calibration standards, preferably measured with replicates. Ideally, the concentrations of the two standards must define an interval including all the analyte concentrations of the sample test solutions [2]. If the methods have no background signal, a calibration with only one standard is possible [9]. This methodology assumes that the intercept of the calibration curve is zero and that the change in the response is directly proportional to the change in analyte concentration. It is mandatory to check that these conditions hold before applying this scheme.

14.2.4 Matrix-Matched Calibration

External calibration is suitable for analytical methods that could be considered free of a matrix effect. When matrix effects are suspected, more reliable calibration may be obtained with calibration

standards containing the analytes of interest and the principal compounds characterizing the sample matrix. Matrix-matched calibration is then a particular type of external calibration where the standard solutions provide a better match to sample composition. In any case, the standard must be representative of the sample.

Standard solutions must be prepared from matrix materials free from analyte and characterized for their composition of specified major, minor, and trace constituents. They may also be prepared by means of synthetic mixtures. The establishment of a matrix-matched calibration is often possible when the analytes are exogenous sample components. For this reason, this methodology is frequently used for pesticides or drug residue analysis [10]. When the matrix effect is sample dependent, it is necessary to use standard additions calibration.

14.2.5 Standard Additions

The standard additions method is an old method. It aims to provide the right determination of one analyte concentration even if matrix effects are present. It involves the addition of increasing the amounts of analyte to the sample itself to compensate for the effect of coextractive compounds on the measuring system. The spiked samples are next effectively measured. From the data obtained, a linear function is calculated. It is possible to quantify the analyte amount initially present in the sample by extrapolation of this expression at signal zero. The various elements that underlie the principle of the method are shown in Figure 14.2.

An interesting comparison between external calibration and standard additions calibration, concerning the analysis of physiological amino acids, has been given by Fernandez-Figares et al. [11]. Other practical uses of the standard additions methodology in phytochemical analysis concern the determination of lignans [12] or hormones [13].

Standard additions calibration is usually carried out with two or three additions, requiring three or four measurements. Rigorously, a calibration for each sample is needed, which implies a lot of work and is very time-consuming. This is a serious drawback to its routine application. To avoid this problem, standard additions calibration can be carried out once for specific samples with similar matrices that have the same behavior in the measurement system. The standard additions calibration curve becomes a matrix-matched calibration characterized by a linear curve but higher intercept. It cannot be extended to the analysis of another matrix type**.** However, this methodology can be used only when the measured signal is specific to an analyte. If the signal is nonspecific (i.e., chromatographic interferences caused by overlapping peaks), systematic error will occur and sully the final result.

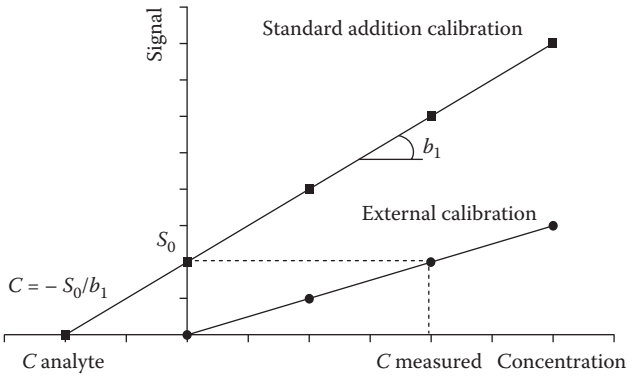

FIGURE 14.2 Standard additions method. The signal S_0, with the traditional calibration curve, leads to a measured concentration two times larger than reality.

14.2.6 Internal Calibration

In some cases, the availability of suitable standards for quantification is limited, and sometimes it is impossible to acquire analyte standards. This problem can be overcome by synthesis of the relevant compounds or by preparative chromatography that isolates the compounds. However, there are some analytical situations in which an internal standard (IS) is added to the sample as a known amount. The signals of the IS and analytes are measured together in the chromatogram of the test sample. From the IS peak, a response factor is obtained that is applied to all the analyte signals for quantification. Quantification is then carried out using this appropriately selected reference compound.

The IS is usually selected to be closely related to the analytes and to represent their analytical behavior the most. It should be distinct from the analytes in order to be quantified separately. It should not interfere with any components present in the sample. Therefore, similarity between the calibration factors of the analytes and the IS is required. It must be verified, or else significant errors in quantification can be found.

An exclusive advantage of this methodology is that it is possible to carry out the quantification of several analytes (of the same family) in the same sample, reducing analysis time. The main limitation is the availability of an adequate IS. Internal calibration is certainly the most used method for the determination of metabolites in plants such as steroids [14], carotenoids [15], flavones [16], or phenols [17].

14.2.7 IS Normalization

Use of an IS also permits one to compensate for matrix effects. For example, with mass spectrometry, the resulting ion suppression or enhancement has a profound influence on accuracy and precision when it comes to quantification of trace levels. Normalization of the measured analytical signal is carried out by spiking a known amount of IS on the calibration solutions and samples. The same amount of IS is added to each one. The multiple external standards measurements are used to determine calibration factors for all components and for the IS. Next, the samples are analyzed, and the analyte contents are corrected based on the relative response of the IS. The method is based on the invariance of the ratio of the calibration factors observed for analytes and the IS. It assumes that a proportional matrix effect changes the signals from the analytes and the IS in the same degree. The use of isotope-labeled analyte as an IS is a powerful way of compensating for matrix effects during the ionization process in the ion source of the mass spectrometer, ensuring accurate and precise results [18].

This method can also overcome losses during the whole analytical process [19]. IS normalization is thus recommended when the sample preparation is complex or variable. If the IS is added just before measurement, there is no compensation for the preparation of the sample but just a correction of uncontrolled analytical signal variations in the measuring system. This methodology is a double calibration, external and internal. Several practical uses can be found concerning the determination of alkaloids with pharmaceutical interest [20,21].

14.2.8 Multivariate Calibration

Generally, in chromatography, data are analyzed as a univariate response, such as the area of a single peak at a single wavelength. The complete resolution of the peak of interest is, in some cases, impossible to obtain, generating matrix effects. However, multivariate calibration at multiple wavelengths is possible. The theory of this methodology is described in some reviews [22,23].

The goal of building a multivariate calibration is to calculate the concentration of the analyte of interest (C value) in unknown samples as a function of several chromatographically determined responses (S values). This methodology involves the individual signals of all the compounds that contribute to a sample's signal. The first step is then to determine which wavelengths should be used to record spectra of pure compounds and suspected interferents. For this reason, the standard

solutions used to establish the calibration model can contain 10 or even 100 substances. The contribution of interferents is then included in the mathematical model to express the response of the instrument. Multivariate calibration is considered difficult because the *S* variables are often impossible to specify before experimentation.

This technique is applied generally when physical information from spectra is available (UV/visible, infrared, Raman, nuclear magnetic resonance, mass spectra). Some practical uses were described recently for plant sample analysis with liquid chromatography [24,25].

14.3 METHOD VALIDATION

The validation of a new analytical procedure takes place after its development and before its introduction into routine use. According to the ISO 17025 guideline, validation is the confirmation by examination and provision of objective evidence that the particular requirements for a specified intended use are fulfilled [26]. In plain language, validating is proving that the method is adapted to its objectives. Concretely, the validation procedure must respond to the following questions: Which analytes can be determined? In which matrices? In which concentration range? At which level of accuracy?

The goal of the method validation is thus to establish documents that will contain a description of the method, the numerical results of a set of experiments, and the complete statistical calculations of data to establish the performance characteristics and the relevance of the method. At the end of the validation protocol, it is possible to know whether the method is acceptable or not. The analyst can refer to many publications describing the parameters of validation to be tested and a selection is given in the bibliography [27–31]. One can distinguish between intralaboratory validation and interlaboratory validation, which is harder to do. The major criteria for an intralaboratory validation are summarized in Table 14.2. It is also necessary to have methodologies that present how to calculate performance characteristics and rules for making the final decision about the validity of the analytical procedure. The reader is invited to refer to specialized works for more information concerning the statistical aspects.

However, there are three important rules to know:

i. The whole method must be validated. Quite often, only the final determination is validated. However, the sample-preparation steps such as extraction must also be validated.
ii. The method must be validated over the whole range of concentrations. A method may work very well at high concentration but be inadequate at low concentrations.
iii. The method must be validated over the whole range of matrices studied.

TABLE 14.2
Usually Accepted Validation Criteria

Criterion	Remarks
Scope of method	Which analytes, which matrices, which equipment?
Response function	Calibration curve/linearity; sensibility
Limit of detection	The lowest concentration of analyte that can be reliably detected
Limit of quantification	The lowest concentration of analyte that can be quantitatively determined with an acceptable level of precision
Specificity/selectivity	Interferences/matrix effects
Trueness/accuracy	Reference materials, recovery test, proficiency scheme, alternative method
Precision	Repeatability/intermediate precision/reproducibility
Dosing range	Which concentration range?
Ruggedness/robustness	Capacity of the method to remain unaffected by small variations in operating parameters

14.3.1 Scope of the Method

First, the scope of the method should be defined. It includes:

i. Analyzed compounds
ii. Nature of matrices
iii. Type of equipment used (HPLC, column, detector, and so on)
iv. Nature and purity of reagents used
v. Type of analysis: qualitative or quantitative

The definition of the applicability depends entirely on the analyst and the knowledge acquired during method development. Analytical instrument validation usually precedes method validation. The procedures are generally provided by the manufacturers and can be covered by a maintenance contract. This aspect of validation is often ignored by analysts. Sometimes, instruments do not fulfill the technical specifications of the users.

14.3.2 Response Function/Linearity

The response function of an analytical procedure is the existing relationship between the response (signal) and the concentration (amount) of the analyte in the sample. The response function can be linear. However, to force a linear function systematically can be irrelevant and may lead to large errors in measured results. In some situations, a nonlinear model induced by the detection method (mass spectrometry, evaporative light scattering detector) or by a wide concentration range can be observed [32]. A second-order or third-order polynomial curve is sometimes needed. Inversely, if the method is used for trace levels analysis, there is no need to test the linearity over the full dynamic range of the instrument.

14.3.3 Limits of Detection and Quantification

The limit of detection (LOD) is the lowest concentration of analyte that can be reliably detected or distinguished from blank. The limit of quantification (LOQ) is the lowest concentration of analyte that can be quantitatively determined with an acceptable level of precision and accuracy. They characterize the measuring instrument more than the method.

An experimental design to characterize the calibration curve as well as the LOD and LOQ can be used [33]: It is recommended to prepare at least five different standard solutions to measure the instrumental response. The levels of concentrations must be regularly distributed in the entire selected working range. For each level, five replicates are prepared, starting from a standard of definite purity. Each repetition is made on an independently prepared solution to respect the independence of measurements.

The principle of the test consists in checking that the share of the variance due to an error in the model is not higher than the variance of the experimental error. The statistical formulas of computation must be adapted to the model (linear, polynomial). In the case of linear model, measurements obtained can be used to calculate the sensitivity (slope) b_1 and its standard deviation sb_1, as well as the blank (intercept) b_0 and its standard deviation sb_0. These standard deviations also make it possible to define the LOD and LOQ as:

$$\text{LOD} = (b_0 + 3 \times \text{s}b_0)/b_1$$

$$\text{LOQ} = (b_0 + 10 \times \text{s}b_0)/b_1$$

LOD and LOQ can also be calculated from a minimum of 10 independent sample blanks with the mean of instrumental response b_0 and its standard deviation sb_0. Another approach to estimate

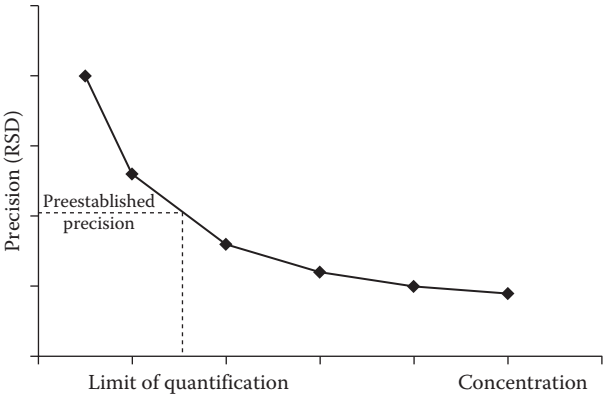

FIGURE 14.3 Determination of limit of quantification. Concentration at required precision is the limit of quantification.

the lower LOQ can be used [34]. The calculated relative standard deviations (RSD) of measurements, which define precision, are plotted against concentration levels close to the LOQ, and a curve is fitted to this plot. When the curve crosses the previously defined required precision, the corresponding concentration level is equal to the LOQ (Figure 14.3).

14.3.4 Specificity/Selectivity

The validation procedure should confirm that the analytical method is able to determine an analyte without interference from other components present in the sample (impurities, degradation products, matrix components). The terms *selectivity* or *specificity* are often used. The International Union of Pure and Applied Chemistry (IUPAC) states that *specificity* is the correct and preferred term if a method is free from interferences [35].

From a chromatographic point of view, the term *selectivity* is often preferred. It refers to a method that may distinguish the responses of a number of chemical entities from each other in a complex mixture [36]. The degree of selectivity depends on the affinities of analytes and other compounds with both stationary and mobile phases and the use of a detector adapted to the analyte properties. It is clear that, in separation technique, a specific analytical method, based on the absence of interferences, must first be selective.

A relevant technique to highlight the matrix effects is the standard additions method. An experimental design to evaluate the specificity can be the following [33]: One addition is carried out with a pure standard solution on several selected samples (minimum 10) with known concentrations of analytes and representative of the applicability of the method. It is advised to choose an addition of the same order of magnitude as the concentration value of the sample being spiked. The most concentrated samples must be diluted to remain in the dosing range of the method. The specificity may be demonstrated if the response of the measuring instrument for the spiked samples corresponds to the added quantity. It is advisable to carry on a diagram the values of additions and the values found and to calculate the straight regression line. The slope must be equal to 1 and the intercept must be equal to 0.

14.3.5 Dosing Range

For any quantitative method, it is necessary to determine the range of analyte concentrations over which the method may be applied. This dosing range can be defined as the interval between the upper and the lower concentrations of analyte for which it has been demonstrated that the analytical procedure has a relevant level of accuracy and precision. Traditionally, in chromatographic

analysis, the concentration range is selected to have a linear relation between the concentration and the signal. The lower limit is then the LOQ and the upper limit is the limit of linearity. When a nonlinear relationship exists, the dosing range can be defined as the concentration interval over which the total error of measurement—or accuracy—is acceptable. It is essential to demonstrate the accuracy of the results over the entire range.

14.3.6 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under stipulated conditions. Precision measures only the distribution of random errors linked to the analytical procedure and does not relate to the true value. The measure of precision is usually computed as standard deviation or relative standard deviation of the test results and is divided into three levels: repeatability, intermediate precision, and reproducibility [37].

Repeatability expresses the precision when the analysis is performed in one laboratory by one operator using one piece of equipment over a relatively short interval of time. Repeatability can be calculated with three concentration levels over the full range of the analytical method (low, middle, and high) with a minimum of three replicates for each concentration. For environmental and food samples, the precision is very much dependent on the sample matrix and on concentration. It can vary from 2% to more than 20%.

Intermediate precision has been defined as the long-term variability of the measurement process. It expresses, then, the within-laboratory variations of results obtained from different days, different analysts, and different batches of reagents. To obtain an estimate of the intermediate precision, it is important to perform the series of experiments in conditions as representative as possible of routine practice, for example, by analyzing systematically an internal control sample in every batch of samples. Intermediate precision can be calculated from data measured on a control sample and monitored on a control chart.

Reproducibility represents the precision obtained between laboratories with different operators and using various equipment (HPLC system, column, and so on), and various operational conditions (reagents) and environmental conditions (room temperature). Reproducibility is determined during a collaborative trial (or interlaboratory validation).

14.3.7 Accuracy/Trueness

The first aim of any quantitative analytical method is to be able to quantify as accurately as possible each analyte concentration in unknown samples. The analytical procedures generally include stages of extraction, purification, and separation followed by instrument calibration and a final detection. Errors can occur at each stage (losses of analyte, contaminations, or interferences), contributing to the bias of measurement. That can be illustrated as:

$$C = c + \delta + \varepsilon.$$

The true value C is estimated by c, which differs from it by the bias (or systematic error) δ and a random error ε. These parameters are inherent in each analytical method [38]. The random error is considered to be normally distributed around x with a standard deviation σ as indicated in Figure 14.4. These errors must be identified and minimized as much as possible.

The trueness of an analytical procedure expresses the closeness of agreement between the mean value obtained from a series of measurements and the value that is accepted as a conventional true value [37]. In the application of this concept, c (and δ) will be obtained by measuring a large number of samples for which the true value of the analyte concentration is known. However, the

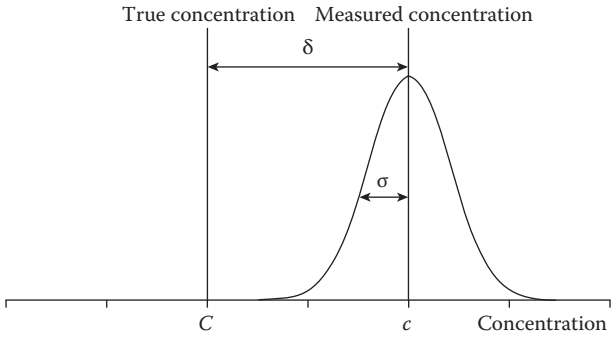

FIGURE 14.4 Representation of the bias (δ) and random error (2σ) in a measurement result.

measurement of trueness cannot distinguish between systematic and random error. One cannot compare a single measurement to a reference value without dispersion of the results.

In ISO documents, the accuracy of an analytical procedure expresses the closeness of agreement between a test result and the accepted reference value. The closeness of an agreement observed is the result of the sum of the systematic (δ) and random (2σ) errors. The accuracy is then the expression of the sum of the trueness and precision. Accuracy can be estimated from reference materials (RMs), interlaboratory comparison (proficiency test), recovery of the spiked amount of analyte added to test samples, or comparison of results with those of an alternative method. Practically, it is through the list of samples selected for the tests that the analyst can show that the applicability of the method is correctly covered in terms of types of matrices and ranges of concentrations. The number of samples can be at least 10, and the number of repetitions at least three.

14.3.7.1 Reference Materials

Accuracy can be assessed by analyzing a sample containing analytes with known concentrations, for example RMs, and comparing the measured value with the true value supplied with the material. RMs are usually real-work materials containing the analytes of interest in their natural form. Appropriate RMs must be accompanied by a certificate that establishes traceability to an accurate realization and each certified value with an uncertainty at a stated level of confidence. RMs should be chosen to have a matrix composition closely similar to the sample matrices. In addition, they should contain the analytes of interest at levels of concentration that are similar to unknown samples. RMs should be analyzed according to the same procedure as the sample, but it is necessary to specify the number of replicates selected.

14.3.7.2 Recovery

In many cases, RMs are not available. In these conditions, the recovery test can be used to evaluate the accuracy of the method. It consists in adding a known amount of analyte to a sample composed of the matrix of interest. The sample is extracted and analyzed with and without the addition to allow the estimation of accuracy.

Accuracy should be reported as the assay's percentage of recovery of a known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals. The additions can be carried out at three levels of concentration corresponding to the range of validity of the employed method. The acceptability of an output of recovery will depend on the type of analyzed substance. For major elements, recoveries of 100% are current in the majority of matrices. In the case of pesticides, values of 50% are frequently observed on environmental matrices.

A recovery test is thus an estimation of the accuracy of the global process but does not consider whether the error is a consequence of changes in the amount of analyte or is due to the matrix effect.

In fact, an addition will never represent the state of adsorption of a substance naturally present in a sample (incorporation in the matrix and chemical bonds with the components of the matrix). A rate of 100% can thus not represent the real extraction of the substance in the sample. Inversely, if a rate of 10% is observed, it is possible to consider that the method of extraction used is not applicable to the analysis considered.

It is also necessary to point out that the recovery testing of an analytical method should be distinguished from the specificity testing. In specificity testing, the aim of the investigation is to verify that the final determination of analytes is relevant without a matrix effect. The additions were carried out after extraction. In the recovery study, the aim of the investigation is to determine the relevance of the whole analytical procedure. The additions are carried out before extraction. The same spiking addition cannot be used both to fulfill the role of calibration and to provide an independent test of accuracy or a specificity test.

14.3.7.3 Alternative Method

Another approach to check accuracy is to compare the results of the method with results from an established reference method known to be unbiased. This approach also assumes that the uncertainty of the reference method is known. Ideally, the analysis should be made using a method based on physical or chemical principles completely different from that used initially. If various procedures give the same results, more confidence can be placed in the method.

The principle of the test (Cochran test) consists of first comparing the standard deviations of the method with those of the reference method, checking the stability of fidelity. Next, the accuracy is established by comparing the differences between means obtained for each sample by each method with the standard deviation of these differences. Rules of decision allow one to predict whether the two methods have the same accuracy.

14.3.7.4 Proficiency Tests

In proficiency testing programs, the performance of a laboratory is evaluated objectively by comparing its results with those of other laboratories. Samples are distributed to laboratories by external organizations, at regular times, for analysis. These organizations are responsible for the design of the scheme, the preparation and the distribution of samples with instructions to the participants, the collection and statistical analysis of the data obtained from the tests, and the feedback of results to participants [39,40]. The proficiency tests focus, then, on the determination of the bias of the individual laboratory's methods, on several matrix types. The data provided by the proficiency test are currently used to calculate the difference between a laboratory's result and a consensual value, established by taking the average of the whole of the results, then comparing this difference with the dispersion of the results (*Z*-score):

$$Z = |c - C|/s,$$

with c the result obtained by a laboratory, C the assigned value, and s the standard deviation between laboratories. The *Z*-score is interpreted as follows:

$Z < 2$ satisfactory performance
$2 \le Z \le 3$ questionable performance
$Z > 3$ unsatisfactory performance

14.3.8 Interlaboratory Method Validation

Interlaboratory validation is determined during a collaborative trial. The objective is to verify that the method provides the same results in different laboratories. If the purpose of proficiency testing is the evaluation of laboratory performance, the purpose of a collaborative trial is often to determine

the precision (repeatability and reproducibility) of a specific method. It is much less applied for the evaluation of bias.

According to the ISO 5725, a collaborative trial requires an experimental design involving one or several test materials, the participation of at least eight laboratories reporting valid data, and most often including replicates (three to five) to assess within-laboratory repeatability parameters [30,41]. The variation within the sample due to homogeneity and stability of its components must be tested previously and must be negligible in comparison with variation between the participant results. The results are then compiled into one large data set to enable a multivariate evaluation. The statistical outliers should be detected and subsequently rejected. Examples of protocols can be found in the literature [42,43]. The ideal validated method has progressed totally through a collaborative study.

14.3.9 Acceptance Limits

The aim of the validation phase is to guarantee that the analytical method will provide, in routine, measurements close to sample true values. This objective cannot be simply limited to obtaining estimates of bias and random error but must be focused on the evaluation of risk. Most of the regulatory documents do not make any recommendation on how to decide when an analytical procedure is acceptable. It is the laboratory's responsibility to justify the decision.

As already mentioned, the difference between a measurement x and its true value X is composed of a systematic error (δ) and a random error (ε). The acceptance criteria may be based on the use of the null hypothesis:

$$C - c = \delta = 0.$$

The decision is based on the computation of the rejection criterion of the Student's t-test. For the given estimates of bias and standard deviation, a procedure is usually declared adequate when a 95% confidence interval of the average bias includes the value 0. Consequently, the smaller the variance, the less likely the confidence interval is to contain the 0 bias value, leading to a rejection of the procedure. On the other hand, the worse the variance, the more likely the confidence interval is to obtain the 0 bias value. This situation is then paradoxical, and this test is inadequate in the validation context [44].

For this reason, the acceptance criterion can be established using a previously defined acceptance limit. In other words, the analyst must prove that the difference between the measured value (c) and the true value (C) is lower than a level of confidence λ:

$$C = c \pm \lambda.$$

The value of the acceptance limit is not arbitrary. λ may be the uncertainty that characterizes the dispersion of the values and that could be attributed to the result of measurement with a defined probability [45]. The uncertainty evaluation leads to the standard uncertainty $u(x)$, which is currently expressed as a standard deviation. Next, λ is obtained by multiplying $u(x)$ by a coverage factor k with a specified probability:

$$\lambda = ku(x).$$

For $k = 2$, the uncertainty corresponds to half the length of a 95% confidence interval. λ may also depend on the requirements of the analyst and the objective of the method. The limit is linked to the requirements usually admitted by the practice [46]. A relative acceptance limit of 15% is a classical choice for bioanalytical procedures. A measurement result cannot be properly interpreted without evaluation of its uncertainty. Results presented without uncertainty are meaningless.

14.3.10 Uncertainty Estimation

Estimation of measurement uncertainty has become a key requirement when a laboratory wants to obtain an accreditation. The *Guide to the Expression of Uncertainty in Measurement* (*GUM*) published by the ISO [47] and interpreted by EURACHEM for analytical chemistry [48] establishes rules for evaluating and expressing uncertainty for a wide range of measurements. This approach requires the identification of all possible sources of uncertainty associated with the procedure [49]. Each individual contribution must be expressed as a standard deviation from experimental data, and the final combination of these individual uncertainties is calculated by using the appropriate rules for error propagation to give the expanded uncertainty for the procedure.

For a typical HPLC method, the sources of uncertainty can be identified as repeatability, bias, measurement of the peak area, concentrations of standards, and the mass and volume of the sample. In the majority of cases, bias and precision account for major contributions (80%) to the total uncertainty [50]. However, contributions associated with sampling are generally excluded. Laboratories are usually not responsible for primary sampling in the field, but they have to prepare the sample delivered to take a representative subsample for testing. Uncertainty associated with sampling depends on three main attributes: the degree of heterogeneity of the sample, the size of the sample, and the method of taking the test portion from the sample [51]. It is not easy to include the uncertainty estimation of sampling process in the metrological model from the *GUM*.

A valuable source of knowledge in the uncertainty for the whole procedure is intermediate precision. Intermediate precision reflects intralaboratory variation to a very large extent, including sample homogeneity. Comparison between the values obtained following the *GUM* model and the overall method performance characteristic has been published [52]. It shows that the two approaches lead to comparable uncertainty estimates.

It is also possible to use a method called the "accuracy profile" of the analytical procedure. The aim of this strategy is to calculate an interval that contains a given probability of future results and check if it fits into the acceptability limits. The description of this concept and applications are given in recent publications [53,54].

14.3.11 Ruggedness/Robustness

Among the other tests of the validation procedure, the robustness (or ruggedness) test is often cited [55]. The more currently applied definition is that the robustness/ruggedness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters. Initially, robustness testing was performed when a method was transferred to another laboratory. The changes introduced helped identify the parameters that might influence the reliability of results. For this reason, the robustness test has become a part of method validation to describe the ability to reproduce an analytical method in different laboratories or in different circumstances [56].

The practical execution of a ruggedness test requires the introduction of small variations in the operating conditions for the method. For an HPLC method, some quantitative factors are the mobile-phase composition and pH, column temperature, or flow rate. Responses should represent quantitative aspects of the method such as retention times, numbers of theoretical plates, resolutions, or peak asymmetry. The factors are usually examined at nominal level [0] and at two levels, [–1, +1], symmetrically around the nominal level. The use of an experimental two-level screening design is recommended, such as fractional factorial or Plackett-Burman designs, examining f factors in minimally $f + 1$ experiments. It is advised to execute the experiments randomly to minimize uncontrolled influences such as a drift or time effect on the results. An estimation of factor effects can be found with a statistical ANOVA approach. A method is considered robust when no significant effect is indicated on the validity of the provided results. Numerous examples of practical robustness tests are available [57–59].

From an analytical point of view, ruggedness/robustness is a measure of intermediate precision or reproducibility. In fact, these two parameters depend on the stability of the analytical chromatographic system (eluent composition, flow rate, detector response). Moreover, according to the U.S. Pharmacopoeia, "the ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same sample under a variety of normal test conditions, such as different laboratories, different analysts, different instruments, different days [55]."

Therefore, chromatographic parameters with a large influence on the instrument's response are generally identified during the method development. If the method is not robust, it will be very difficult to validate it. The robustness test is time-consuming and is advantageously replaced by reproducibility or intermediate precision.

14.4 QUALITY ASSURANCE/QUALITY CONTROL FOR HPLC METHODS

Quality assurance (QA) is the overall organizational system that forms the basis for all reliable analytical measurements. QA should involve all the steps of chemical analysis as an integral process (Figure 14.5). QA thus includes staff formation, maintenance and metrological calibration of all equipment used, the laboratory environment, and a system of sample identification, record keeping, and storage. Traceability is the foundation of any QA system [60].

Quality control (QC) is defined as the practical activities that establish that an analytical method, at any given moment, is able to perform routine analysis according to its design specification [61]. QC defines the range of circumstances when a set of results can be accepted. QC implies the daily quantitative evaluation of a measuring system, such as calibration, accuracy, and precision. If the performance of the system is not suitable, the acceptable operation conditions have to be restored before starting and continuing analysis.

Several reference tests exist such as Good Laboratory Practice and the QA systems ISO 9001 and ISO 17025. However, these international standards provide only general rules for installing and maintaining a quality system. It remains the responsibility of the laboratory to define appropriate procedures that assure that adequate quality is achieved and to spot changes in the performance of the method used [62].

The application of a quality system in liquid chromatography can be based on the use of blank, calibration, and control samples [63,64]. Blank and calibration samples allow monitoring of the measuring system. Control samples allow control of the whole analytical procedure including the sample-preparation step. Obtained results must be fully documented, especially with control charts, and stored for later inspection. Therefore, by analyzing control samples at regular times, the long-term reliability of the system is established, and confidence in the results produced is generated.

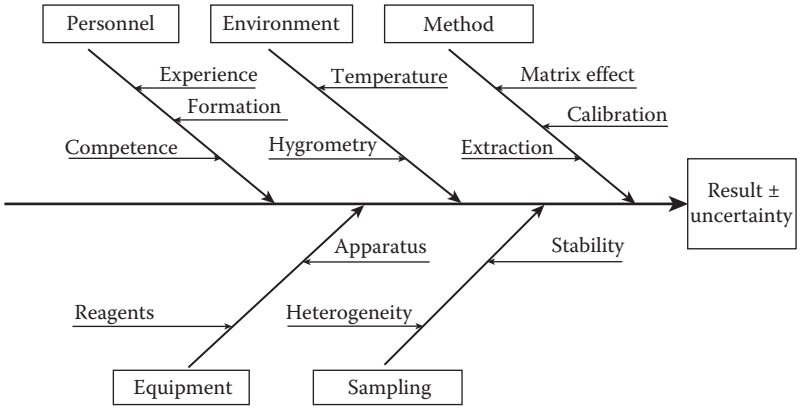

FIGURE 14.5 Analysis of the measurement process.

14.4.1 Use of Control Charts

The general use of control charts is the most widespread procedure to establish an internal quality system for a quantitative method [65]. In control charts, the variable of interest is usually plotted on the y-axis versus time or sequential run number. The new control values are collected as part of routine work and are added to the control charts. Based on the data collected during a certain period of time, acceptable performance limits can be established to ensure reliable results. It is useful to insert on the chart some lines corresponding to the expected value, the warning limits (two standard deviations), and the control limits (three standard deviations), which correspond to the confidence intervals of 95% and 99.8%. These critical values are generally calculated from at least 15 sufficient stable observations [66].

The analyst monitors the working parameters of the HPLC method in order to assure that the results are in the range established by the prescribed tolerance limits or to detect deviation from statistical control. The following indicators have been proposed as the most useful [67]: When the new control value falls within the control limits, the run can be classified as in-control, and the analytical result can be accepted and reported. When the new control measurement value falls outside the control limits, the run must be classified as out-of-control, and the analytical result should be rejected and not reported. One result may fall outside the warning limit and require no action, provided that the next result falls inside them. The observation of two-consecutive results outside the warning limits indicates the need for remedial action.

14.4.2 Instrument Qualification

The instrument qualification supplies necessary information for knowing how the measurement process is working. Test parameters for complete HPLC systems are given in Table 14.3. It shows their importance for the quality of the analytical result involved. Testing all these parameters is very time-consuming and should be applied if a defective function is discovered. In addition, analytical procedures involve different steps during sample preparation (extraction, derivatization, and so on). They increase the number of possible sources of error (Table 14.4). For these reasons, a QC procedure with few but relevant tests that control a wide number of variables is better.

14.4.2.1 Blank Injection

A blank sample, consisting of the solvent only, must always be the first sample injected in the HPLC instrument. The resulting chromatogram shows the summation of the instrumental background plus the signal in the analyte peak region of interest due to interfering species. A blank sample is then injected to check for potential sources of contamination due to the chromatographic system (column, mobile phases, derivatization reagents). Ideally, the chromatogram must show only the peak caused by the injection system. However, extra peaks may appear caused by the late elution of

TABLE 14.3
Operating Parameters of the HPLC Instrument That Affect the Quality of Determinations

HPLC Module	Source of Error	Effect
Injector	Injection volume	Precision of determinations, memory effects
Flasks of auto sampler	Cleanness	Contamination
Separation column	Capacity, efficiency, selectivity,	Accuracy or/and precision of determinations
Mobile phase	Retention times of substances	Concentration accuracy, contamination
Flow rate	Stability, pressure	Accuracy or/and precision of determinations
Temperature	Stability	Precision of determinations
Detector	Sensitivity, linearity, noise	Accuracy or/and precision of determinations

TABLE 14.4
Parameters Affecting the Quality of Determinations during Sample Preparation

Parameter	Potential Error
Heterogeneity of sample	Precision of determinations
Storage of sample	Losses of analytes
Grinding of sample	Contaminations and/or losses of analytes
Extraction of sample	Contaminations and/or losses of analytes
Derivatization	Contaminations or/and losses of analytes, accuracy of determinations
Flasks	Contaminations of sample
Choice of internal standard	Accuracy of determinations
Choice of reference materials	Accuracy of measurements
Preparation of calibration solutions	Accuracy of determinations

a component from a previous injection or carryover in the system. This first blank sample can also help to identify a baseline problem. Therefore, the pump pressure and the flow rate can be observed during this run.

14.4.2.2 Standard Sample/Calibration

Before calibration, it may be interesting and fast to test only a simple standard sample prepared by spiking a blank sample with a known concentration of the analytes of interest and an internal standard. A single calibration solution can be used. With this sample, peak shapes, retention times, gradient performance, and sensibility (peak area/height) are evaluated in particular. Changes in the retention times inform the analyst about problems with column temperature, mobile-phase composition, or column degradation. A control chart for analytes' retention times can be a useful tool to follow the column deterioration and to decide when the column needs to be washed or replaced [68]. The plate number for some components can also be calculated and monitored. After only a short testing time, it is then possible to judge the functioning of the HPLC parameters such as sensitivity of detection and separation of critical peaks.

External standard solutions are usually prepared and analyzed for each analytical set. The quality monitoring can be based on distributions of calibration parameters such as slope (for linear calibration). The slopes of the different calibration graphs must not differ significantly from the value found during method validation. However, all calibration graphs are not necessarily linear. In these conditions, the quality monitoring can be based on the coefficients of equation from the calibration data. Bias in the quantitative analysis may be caused by an error during the preparation of standards.

14.4.3 Accuracy

Accuracy is measured on control samples with known compositions that are treated in the same way as unknown samples. The function of these samples is to detect changes in performance during routine operations [69]. The analytical batch should include at least three control samples, preferably at the start, the middle, and the end of the batch. The arithmetic means of the concentration values, calculated on the three replicates, are integrated to control charts. If they are within predetermined limits, the calibration is valid and can be used to quantify an unknown sample. The main difficulty is to select a control sample suitable for measuring multianalytes, at concentration levels within the selected concentration range.

RMs with known concentrations are certainly the most popular tools to prove the accuracy of the whole analytical process [70]. Different matrix samples can be purchased from international organizations (IRMM, NIST). Normally, the certified values are used as the assigned values. For

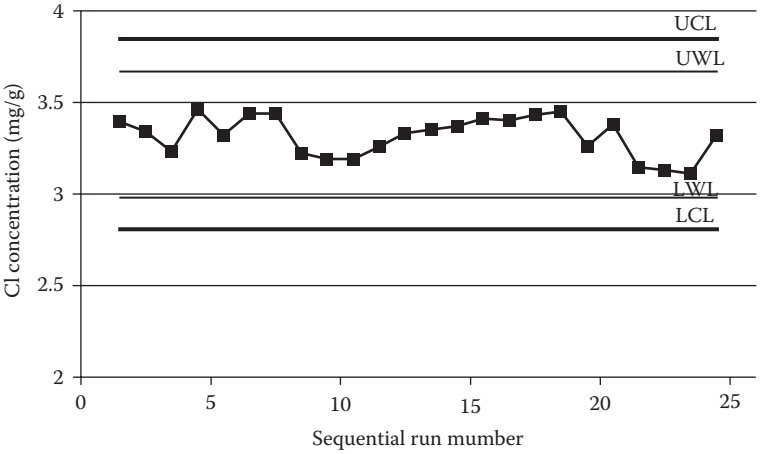

FIGURE 14.6 Control chart of the chloride concentration in a plant sample. Each point is the observed mean obtained on three replicates. UCL, upper control limit (mean of mean + 3 σ); LCL, lower control limit (mean of mean – 3 σ); UWL, upper warning limit (mean of mean + 2 σ); LWL, lower warning limit (mean of mean – 2 σ).

quantitative confirmation procedures, the acceptance range of concentration of the analytes can be established at ± *X*% of the assigned concentration, which can be 5%, 10%, or 20% in agreement with the criterion of accepted uncertainty [71].

RMs remain expensive and are sometimes not easily available for several materials or analytes. In this case, quality control can be performed using an internal control sample with known values. Internal control samples consist of a large amount of material prepared within the laboratory and representative of the real-test material. Natural material is the best choice. It must be prepared, stored, and checked for stability and homogeneity. Concerning its composition validation, the best approach to obtain true values of each single analyte is to use a consensus value produced by a group of qualified reference laboratories [40]. A valid alternative is to use matrices spiked with the substances of interest [72]. After extraction of the analytes from the matrix and injection into the HPLC instrument, their recoveries can be determined by comparing the responses of the spiked extract and of the sample not spiked. The data obtained from the quality control sample are monitored on control charts with warning and control limits. An example is presented in Figure 14.6. The information collected during this stage can be also used to evaluate intermediate precision and uncertainty.

However, an internal control sample can only monitor performance related to variable factors or random error. If the analytical procedure is based on erroneous assumptions, the analytical results will be wrong even if no statistical variations are reported by the QC procedures. In these conditions, participation in aptitude tests (proficiency testing programs) is the way generally followed by laboratories to ensure their analytical quality by an external point of view over time (see also Section 3.7). During recent years, in the field of analytical chemistry, the number of aptitude tests have increased. Therefore, to obtain accreditation, laboratory must demonstrate an effective QC system, which includes participation in relevant proficiency testing programs.

14.4.4 Precision

The measured concentrations from the quality control samples can be used as simple means to check the repeatability of the procedure and to document the functioning of the complete system in terms of stability and precision. In fact, the standard deviation of the measured concentrations is a measure of the overall repeatability of the complete method involving variations of injection, flow rate, and extraction. Comparison of an obtained value with a prescribed uncertainty indicates whether or not

the system fulfills the precision requirements. For environmental and food samples, the precision is highly dependent on the sample matrix and varies from 2% to more than 20%. For drug analysis, in pharmaceutical quality control, a precision better than 1% RSD is easily achieved [44].

QC demonstrates the stability and comparability of measurements over time. However, a chemical analysis chain is only as strong as its weakest link. If elements such as preparation, extraction, or cleanup of the samples have not carefully been carried out, even the best analytical instruments and computer techniques cannot prevent the analytical results from being questionable. The quality of results thus requires knowledge of the whole analytical process, especially for methods that require extraction before the chromatographic run.

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15 Confirmation of Chirality of Some Natural Products by the HPLC Method

Beata Polak

CONTENTS

15.1 INTRODUCTION

Optical isomers (stereoisomers) have been known for many years and were first identify by Biot in the early 1800s [1]. Their existence was established by Pasteur in 1848 [2]. In 1883, Lord Kelvin used the term *chirality*, derived from the Greek *kheir* or *chiros* for handedness [3]. Chirality has very important and essential roles in the existence of the universe (e.g., plants, animals). All living nature is dissymmetric, whereas dead nature is symmetric. Any object lacking the elements of symmetry (i.e., plane, center, and axis of symmetry and helicity) and existing in two forms that are nonsuperimposable mirror images of each other is called a chiral object. Examples of molecules' chirality are presented in Figure 15.1.

A center of chirality (stereogenic center) is an atom to which four different atoms or functional groups are bonded. Mostly, it is a tetrahedral carbon atom with sp^3 configuration; however, other elements also form stereogenic centers. This center can be an atom of nitrogen, phosphorus, arsenic, or sulfur in which a free electron pair exists. The numbers of enantiomers depend on a center's chirality number. The maximum amount of isomers can be calculated according to formula

$$\text{Numbers of isomers} = 2^n, \text{ where } n \text{ is the number of the chirality center.}$$

Helicity is a property of a compound in which groups are spatially arranged along the screw line or their structure resembles a thread. Helical structures are shown by proteins, polysaccharides, nucleic acids, and hexahelicen.

In planar chirality, the asymmetric substitution of groups in a molecule is in relation to a determined plane. An example is the polymethyl ether of bromohydroquinone, cycloalken, and ryfamycin B. In axial chirality, there are compounds with restricted rotation about a single (biphenyls) or double (allens, spiran compounds) bond. An example of this kind of molecule in nature is corilagin (tannin agent), which was isolated from *Caesalpinia coriaria* [4] or gossypol.

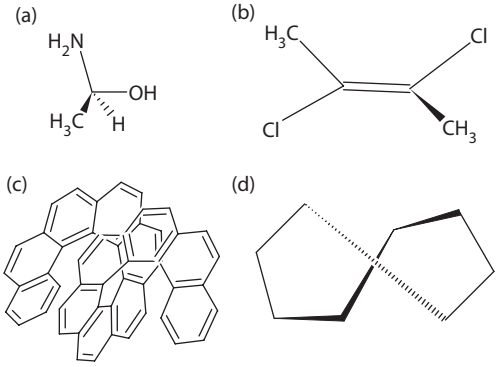

FIGURE 15.1 Examples of molecules' chirality: (a) chiral center; (b) axial chirality; (c) helicity; (d) planar chirality.

The second word connected with chirality also has a Greek origin: *enantiomorph* is the opposite form of subject. Enantiomers have the same physical properties (melting point, boiling point, solubility), the one exception being the various rotations of a polarized light plane. One enantiomer rotates in a clockwise direction (+, dextrorotatory); the second one rotates in a counterclockwise direction (–, levorotatory). Enantiomers relate to each other as an object and its nonsuperimposable mirror image. They show various properties in chiral environments. Any biological system may be considered chiral and can distinguish one enantiomer from another. Carvone (5-isopropenyl-2-methylcyclohexen-2-on) is a constituent of various volatile oils. (4*S*)-(+) - carvone has a caraway smell, while (4*R*)-(–)-carvone smells like spearmint. Also, methyl jasmonates have various odors; (1*S*,2*R*)-(–)-epi methyl jasmonates is odorless, whereas (1*R*,2*S*)-(+)-epi possesses a strong floral odor (like true jasmine) [5]. A system that contains only one form of enantiomer is called homochiral or enantiomeric pure. A system that contains more than one enantiomer in comparison to a second enantiomer is called enantiomeric enriched (enantioenriched). A mixture of an equal amount of enantiomers is a racemate, and its optical activity is 0. The solubility of a racemic mixture differs from that of an enantiomer.

Diastereomers are a second group of stereoisomers. They contain more then one chiral center and possess the same sequence of substituents at one asymmetric atom. Diastereomers are not their mirror image. They have various physical and chemical properties. Therefore, they can be separated by different methods such as achiral liquid or gas chromatography, crystallization, and so forth. For example, the melting point of diasteroisomers of chloromalic acid is 145°C and 157°C; the rotation of the polarized light plane is 7.1° and 9.3° [4].

Natural compounds usually exist in one enantiomeric form, rarely in enantiomeric excess (as gossypol in cotton seeds). However, the racemization process is also occurring, especially during ripening (as naringin in citrus fruits [6]) or product storage [7].

The determination of an optical isomer may be possible by forming the diastereomeric form or without this procedure. The determination without forming diastereomers occurs when the rotation of the plane of polarized light by the isomer is measured (in polarimeters). Also, it is possible to measure the absolute configuration by the crystallography process (as for alkaloids in *Stephania* species [8] or diterpenoids in *Salvia* species [9]) or coupling crystallography with nuclear magnetic resonance (NMR) [10].

The absolute configuration of chiral compounds can be also determined by utilizing special detectors (circular dichroism detector [CDD], vibrational circular dichroism [VCD] or mass spectrometry [MS]) that work on-line with HPLC equipment. An example of such analysis could be gossypol analysis [11] or tropane alkaloids [12]. A system of several detectors (MS-NMR-CDD) (CDD: circular dichroism detector) with HPLC was used by Bringmann and Lang to determine the absolute configuration of natural products [13]. Broad reviews of methods of absolute configuration determination were recently written by Allenmark and Gawroński [14], Bringmann et al. [15], Polavarapu [16], and Roussel et al. [17], as well as authors of the book *Chiral Analysis* [18].

The separation of enantiomers is very difficult. The first method of racemate separation was developed by Pasteur. It was a recrystallization method and was utilized to isolate tartaric acid enantiomers. The crystals of both enantiomers of sodium ammonium salt took different forms, and these various crystals were separated by Pasteur using a magnifying glass [19]. Today this method of separation is still used. However, the basis of separation is the forming of various covalent compounds or salts with homochiral compounds. Next, these diastereomeric mixtures are recrystallized such that one diastereomeric mixture is solid out of solution, and the other one is still dissolved. This method can be used for separation of carboxylic acids (forming diastereomeric salts with chiral amines or alkaloids) or alcohols (first forming phthalic monoesters, then reacting with amines) [20].

15.2 MECHANISM OF CHIRAL SEPARATION

In HPLC the determination of compound configuration relies on the separation of enantiomers in the mixture and comparison to the model compound. Two chromatographic factors describe enantiomer separation:

1. Enantioseparation factor, α, ($\alpha = k_2/k_1$), where k_2 and k_1 are the retention factors of the second and the first eluted enantiomer
2. Peak resolution, R_S ($R_S = (2(t_{r2}-t_{r1}))/w_{b1}-w_{b2}$); where t_r is retention time and w is the peak width

Good peak resolution occurs when α and R_S are bigger then 1.5. The chromatographic methods used to separate racemates are divided into two categories:

- indirect
- direct

In the indirect method, the diastereomeric salt or other diastereomeric compound forms before the chromatographic separation, and then the proper separation process is carried out on common stationary phases. Chiral isothocyanates [21], chiral chlorides, chloroformates, and other chiral compounds can be used as chiral-derivatizing agents (CDAs). Reviews of the indirect separation method have been published in [22–24].

$$\underset{\text{Racemate}}{R,S\text{-compound} + R\text{-CDA}} \longrightarrow \underset{\text{Diastereomer 1}}{R\text{-compound-}R\text{-CDA}} + \underset{\text{Diastereomer 2}}{S\text{-compound-}R\text{-CDA}}$$

The direct method requires a chiral selector chemically bonded to a stationary phase and forming a chiral stationary phase (CSP). The distinguishing of two enantiomers on a CSP occurs when one enantiomer of a racemic mixture has more interactions with the selector in comparison to the second one. The most important difference in interactions is connected with the spatial position of one of the functional groups. This "three-point interactions" rule was introduced by C.E. Dalgliesh [25].

The typical interactions that occur in a chiral separation system are hydrogen bonding, π-complexation, coordinative bond, inclusion complexes, dipole stacking, and others. During the separation process, one interaction is leading, and CSPs are divided into several groups based on this main interaction [26]:

1. Pirkle's CSP—dominant π-complexation
2. Modified carbohydrate-type CSP—main interactions are attractive interaction (hydrogen bonding, dipole stacking) and inclusion complexes
3. Cyclodextrin-type CSP—inclusion interaction inside chiral cyclodextrins' cavities
4. Ligand-exchange CSP—complexation with transition metal ion (mostly Cu^{2+})

5. Mixed phases on which a combination of polar and hydrophobic interactions occurs (immobilized proteins; some polysaccharide phases)

A broad study of chiral molecular interactions can be found in Grinberg et al. [27].

15.3 ALKALOIDS

Alkaloids are plant metabolites that occur in several plant families such as the *Papaveraceae*, *Papilinaceae*, *Leguni*, *Ranunculaceae*, and *Solanaceae*. This group includes about 15,000 compounds. A characteristic feature of alkaloids is that they possess a nitrogen atom or atoms, mostly in a heterocyclic ring, and amino acid or amino acids origin. They are natural bases due to presence of nitrogen atoms. Alkaloids show strong physiological interactions. These compounds usually accumulate in external plant parts such as bark (quinine), leaves (cocaine), and seeds (strychnine, brucine) [28]. The main classification of alkaloids is based on chemical structure, and it is as follows [28,29]:

1. Proper alkaloids (possess nitrogen heterocyclic system)
2. Pseudoalkaloids (compounds possess nitrogen but it does not have amino acid origin)
3. Protoalkaloids (alkaloids without a heterocyclic system, such as ephedrine)

Each of these groups has its own subclassification. Table 15.1 lists examples of natural chiral alkaloids.

The absolute structures have been precisely determined for the majority of common known alkaloids (brucinie, ephedrine, quinidine, quinine). Some alkaloids have been utilized for separation of chiral acids' racemate through the forming of diastereomeric salts [30–32]. Nowadays, after molecular modifications, some alkaloids have been used to form the chiral selectors in chiral HPLC columns, for example, quinine in Lindner's type CSP [33–36]. The chiral determination of alkaloids concerns newly isolated alkaloids. Below there are some examples of determination of chiral alkaloids.

Erbutamine-vincamine alkaloids are a large group of pharmacologically active bases. Due to the presence of three stereogenic centers in molecules of the vincamine group alkaloids, formation of eight stereoisomers is possible. A valuable therapeutic activity in cerebral insufficiencies is exhibited by (+)-vincamine. This alkaloid was first isolated from *Vinca minor* L. *Apocymaceae*. Caccamese and Principato [37] reported the direct separation of the four pairs of enantiomers from the vincamine group on a polysaccharide-derived CSP (Chiralpak AD; column built from amylose tris (2,5-dimethylphenylcarbamate)). Resolution factors (R_s) were satisfactory for all pairs of isomers. The resolution was carried out in normal-phase mode using isocratic or gradient elution. In addition, the chiral recognition model was proposed. The vincamine alkaloids interact with the CSP via a π-π interaction between the indole moiety and the phenyl group of the CSP and via hydrogen bondings.

TABLE 15.1
Examples of Natural Chiral Alkaloids

No.	Alkaloid Class	Example of Chiral Member
1.	Protoalkaloids	Ephedrine
2.	Piperidine derivatives	Koniine, lobeline, sparteine
3.	Noncondensed rings	Nicotine, anabazine
4.	Tropane derivatives	Atropine, scopolamine
5.	Quinoline and isoquinoline derivatives	Quinine, quinidine, cynchonine, cynchonidine
6.	Opium alkaloids	Narcotine, morphine, codeine
7.	Indole derivatives	Strychnine, brucine, vincamine, vincristine, vinblastine
8.	Terpenoid derivatives	Akuamicine, akuamidine, stryctamine
9.	Steroid derivatives	Solanidine, solasodine, solafloridine, conessine

Cularinoids belong to isoquinoline group of alkaloids. They are characterized by a benzoxepine skeleton but occur naturally in various oxidation states. Among them aristoyagonine is a cularine alkaloid with five-membered lactam ring. The resolution of enantiomers of *N*-*p*-methoxyl-1, α-dihydroaristoyagonine, and 4′,5′-dimethoxy-1-α-dihydroaristoyagonine was accomplished using five different CSPs by Caccamese et al. [38]. Better separation was obtained using polysaccharide-derived CSPs. It should be mentioned that, among the columns possessing the same chiral selector, Chiralcel OD-H columns built from cellulose tris (3,5-dimethylphenylcarbamate) afforded minor R_S values in comparison to Chiralpak IB, made of tris (3,5-dimethylphenylcarbamate) derivatized amylase immobilized on the support. Differences between R_S factors were caused by column support. Pirkle-type columns (β-GEM) have also shown good resolution of racemic compounds; however, there have been many disadvantages such as high capacity factors and high elution time. The enantioseparation on polymeric CSPs (Kromasil CHI-DMB; the chiral monomer is *O*,*O*′-bis-(3,5-dimethylbenzoyl)-*N*,*N*′-diallyl-L-tartar diamide) was the weakest. One of these CSP columns was also utilized for the semipreparative isolation of one enantiomer. Other alkaloid enantiomers from the same group of alkaloids—DL-tetrahydropalmitine (THP) and DL-tetrahydroberberine (THB) isolated from *Corydalis yanhusuo*—were investigated on a cellulose (3,5-dimethylphenylcarbamate) (CDMPC) CSP. The chromatographic method was validated and the analysis was carried out in both analytical and semipreparative mode [39].

The method of chiral ligand-exchange liquid chromatography with a Sumichiral OA-6000 column—the chiral selector was (*R*,*R*)-tartaric acid mono(*R*)-1-(α-naphthyl)ethylamine)—and electrochemical detection was utilized to separate synephrine enantiomers (phenylethylamine group of alkaloids), which also exist in food (*Citrus unshiu* fruits, orange juice, marmalade). After validation, the method was also used for determination of conjugated synephrine enantiomers in urine. It was noticed that in vivo the chiral inversion of L-synephrine into D-synephrine occurred [40]. Pellati and coworkers [41] investigated the content of enantiomers of DL-synephrine in *Citrus aurantium* L. var. *amara* products (fresh and dried fruits, dried extracts, and herbal products) using β-cyclodextrin CSP, in reversed-phase mode with isocratic condition and detection at 220 nm. The highest amount of L-synephrine was found in dried extracts. The opposite enantiomer was present in small amounts in fresh fruits and commercial products. In addition, the conversion of L-synephrine to D-synephrine by high temperature was monitored by the same chiral system.

The same alkaloid was also determined on a protein-based CSP (Chiral-CBH, selector cellobiohydrolase) with reversed-phase mode and detection at 225 nm [42]. The analytical method developed was validated and applied to determine the amount of synephrine enantiomers in *Citrus* fruits (see Figure 15.2). The strong cation-exchange solid-phase extraction (SPE) was suitable for cleaning up the investigated extracts before LC analysis.

A review of different methods of synephrine investigation was presented by Pellati and Benvenuti [43]. The chiral analysis was performed using different CSPs (Sumichiral OA, Chiradex, and Chiral-CBH) and reversed-phase conditions. The best results were obtained by Kusu et al. [40], who used the chiral ligand-exchange column Sumichiral OA-6000, built from (*R*,*R*)-tartaric acid mono-(*R*)-1-(α-naphthyl)ethylamine, and obtained good separation results: $\alpha = 1.62$ and $R_S = 2.25$. The presence of the same alkaloid in *Evodia rutaecarpa* and *Evodia officinalis* was investigated utilizing the Chiral-CBH column and reversed-phase mode [44]. The (–)-synephrine was the main constituent in *Evodia rutaecarpa* fruits extract, whereas *Evodia officinalis* produced only (–)-synephrine.

The Chinese plant *Dracocephalum rupestre* contains trace amounts of alkaloids. They belongs to the flavonoidal (conjugates of flavanone with pyrrolidin-2-one) group of alkaloids, and they were named dracocephins. The isolation and determination of four groups of these compounds were done by Lou's group [45]. The separation of chiral dracocephins A, B, C, and D was achieved using the analytical Chiralpak AS-H column (chiral selector is amylose tris [(*S*)-α-methylbenzylcarbamate] coated on 5 μm silica gel) coupled with circular dichroism detector (CDD). The solvent system used for separation of compounds A and C consisted of pure acetonitrile, while that for groups B and D was a mixture of acetonitrile (90%) and 2-propanol (10%). In Figure 15.3

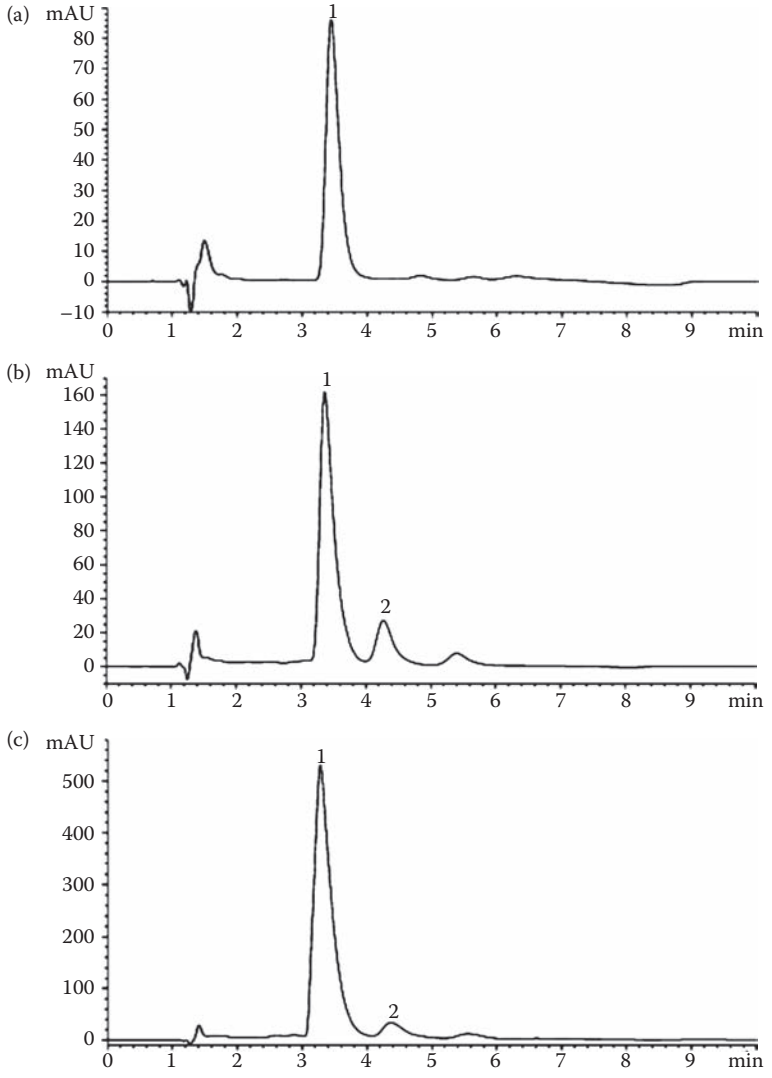

FIGURE 15.2 Chromatogram of separation of synephrine enantiomers (a) from *Citrus aurantium* fruits; (b) from *C. aurantium* dry extract; (c) from dietary supplement. 1, (–)-synephrine; 2, (+)-synephrine. (Reprinted from Pellati, F., Benvenuti, S., and Melegari, M., *J. Pharm. Biomed. Anal*., 37, 839–849, 2005. With permission.)

the separation of dracocephin A is presented, and the four HPLC-UV peaks (bottom line) correspond to the two positive (2*R*, 5"*S* and 2*R*; 5"*R* enantiomer) and two negative (2*S*, 5"*R* and 2*S*; 5"*S* enantiomer) signals in the CD trace (upper line). The order of the separated alkaloids was 1, 3, 2, and 4. The CD spectrum of the stereoisomer allows the determination of the steric structure of the molecules: A1 - 2*R*, 5"*S*; A2 - 2*S*, 5"*S*; A3- 2*S*, 5"*R*, A4- 2*R*, 5"*R*. The steric structures of the second group of dracocephins B, C, and D were determined in a similar mode.

Polysaccharide-derived CSPs (Chiralpak AD, Chiralcel OD) were applied to the separation of some indole alkaloids of *Tabernaemontana eglandulosa* by Caccamese and coworkers [46]. Small structural differences between Chiralpak AD and Chiralcel OD caused better enantioseparation on Chiralpak OA. The good enantioselectivity ($\alpha = 4.39$) and resolution factor ($R_S = 2.8$) allowed semi-preparative separation of tacamonine and vindeburnol. The specific rotation of these two alkaloids was also determined.

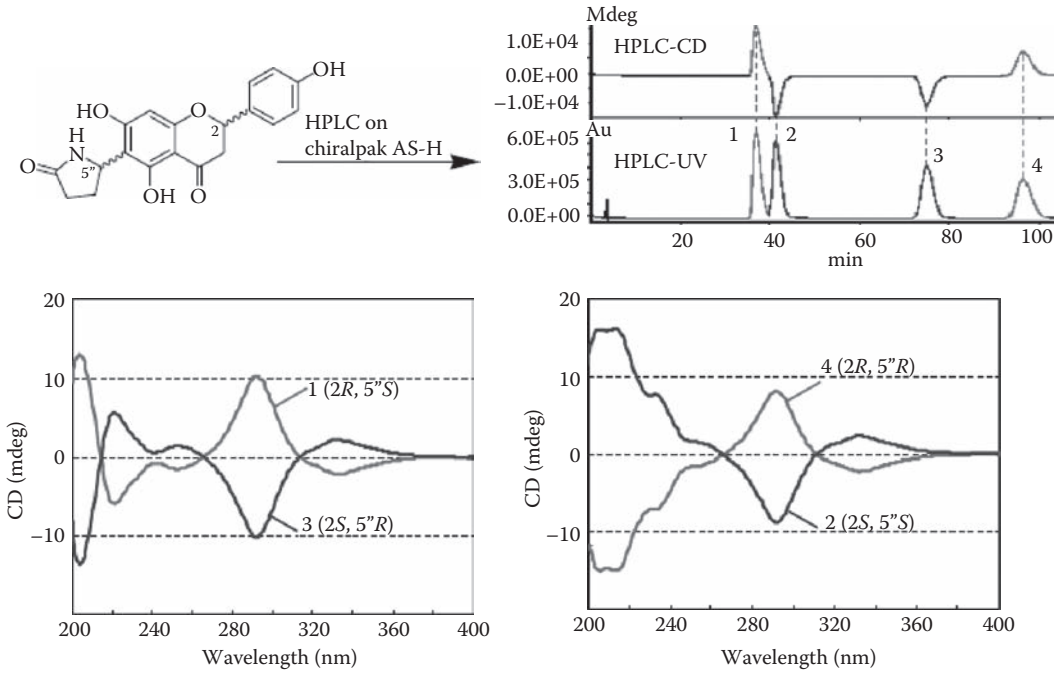

FIGURE 15.3 HPLC-UV and HPLC-CDD (CDD: circular dichroism detection) chromatograms of dracocephin A. (Reprinted from Ren, D.M., Guo, H.F., Yu, W.T., Wang, S.Q., Ji, M., and Lou, H.-X., *Phytochemistry*, 69, 1425–1433, 2008. With permission.)

Isolation, structure elucidation, total chiral synthesis, and analysis of a new Corynanthe-type indole alkaloid 9-methoxymitralactonine were described by Takayama and coworkers [47]. Chiral analysis of natural alkaloids on Chiralcel OD column has shown that the (–) enantiomer predominated in nature over the (+) enantiomer in the ratio 62:38 (see Figure 15.4).

The process of demethylation of tobacco alkaloid enantiomers through the *Nicotiana plumbaginifolia* cell suspension was presented by Mesnard et al. [48]. The worthwhile result was that the plant cells were unable to biosynthesize alkaloids. Both kinds of enantiomers were effectively demethylated in the presence of each other, but rates of degradation were different (nonnatural *R*-nicotine degradation was four times faster). The final products were investigated utilizing the chiral OD-H column with normal-phase mode.

A new naphtholisoquinoline alkaloid, dioncophylline E, was isolated from *Dioncophyllum thallonii* and chromatographically investigated using the Chiracel OD column by Bringmann's group [49]. The same group of alkaloids was isolated from the leaves of the Malaysian highland liana *Ancistrocladus benomensis*. One of them, chiral dioncophylleine A, was successfully separated into enantiomers on a Chiracel OD-H column [50].

The ergotamine alkaloids (lisuride, terguride, meluol, and nicergoline) were investigated using various CSPs with vancomycin and teicoplanin as chiral selectors. The influence of the chiral selector type and mobile-phase properties (type of organic modifier, buffer concentration, and mobile-phase pH) on alkaloid separation was investigated. The best results were obtained for methanol as an organic modifier. Better separation of lisuride was achieved when Chirobiotic V CSP (chiral selector is covalently bonded macrocyclic glycoprotein-vancomycin) was used, whereas for meluol Chirobiotic T (chiral selector is covalently bonded macrocyclic glycoprotein-teicoplanin) provided better results [51].

Tropane alkaloids (atropine, scopolamine) are the main alkaloids in the *Solanaceae* family. Chiralcel OD was applied to the separation of tropane alkaloid enantiomers [52]. The new method

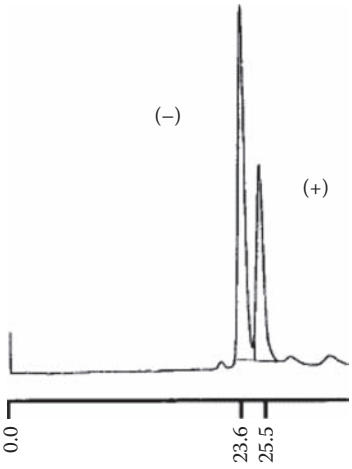

FIGURE 15.4 Chiral HPLC analysis of natural (–)-9-methoxylmitralactonine. (Reprinted from Takayama, H., Kurihara, M., Kitajima, M., Said, I.M., and Aimi, N., *Tetrahedron*, 56, 3145–3151, 2000. With permission.)

can be utilized to determine enantiomer purity of *S*-(–)-hyoscyamine. The tropane alkaloid racemic atropine was separated into enantiomers on an α_1-acid glycoprotein CSP [53]. The chromatographic system was coupled with MS. The achieved results depend on chromatographic conditions such as the organic solvent and pH of the mobile phase.

Cephataxus alkaloids are heterocyclic compounds that show antileukemic activity. They are produced by trees of the genus *Cephalotaxus*. Five alkaloids of this new type were isolated from *Cephalotaxus harringtonia* var. *nana* [54]. Their absolute configuration was elucidated by nuclear overhauser effect spectroscopy (NOESY) data and CD analysis. The sugar unit from one alkaloid (cephalezomine J) was determined chromatographically using the CSP Chiralpak OP (+) the chiral selector is poly(diphenyl-2-pyridylmethymethacrylate).

15.4 FLAVONOIDS AND FLAVANONES

Flavonoids and flavanones are secondary plant metabolites from the shikimic acid pathway. Their skeletons consist of 15 carbon atoms (benzene ring, A-ring, and phenylpropane system B-ring). They can also be determined as benzo-γ-piron or chromon. The basic structures of flavonoids are flavan, flavon, flavanon, isoflavanon, chalcone, flavonol, flawanonol, isoflavanon, auron, and neoflavon [28]. Flavonoids are yellow or colorless solid substances dissolved in cell juice in flowers and leaves, rarely in fruits, bark, and wood. The majority of flavonoids exist as *O*-glycosides and possess a sugar part, which consists of one to five molecules of simple sugars. Sugar units usually exist at the C-7 position on ring A. Sugar moieties of flavonoids have optical activity. Due to this property, the flavanone glycosides possess an additional stereocenter. Almost all the flavonoids have one stereocenter in position C-2 except a subclass of flavanones (3-hydroxyflavanones or dihydroflavanols) that have two chiral carbon atoms at positions 2 and 3. A review of the separation of chiral flavanones was published by Yanez and coworkers [55]. These authors presented the general principles for separating this class of compounds, also in biological samples.

A series of chiral biflavonoids, derivatives of maesospsin, were isolated from heartwood of *Berchemia zeyheri*. All these compounds belonged to benzofuranoid oligomers class and possess the stereogenic center. They were resolved into enantiomers on Chiralcel OD stationary phase [56].

Naringine is a chiral flavanone glycoside. It is chiral due to the presence of a stereogenic center in the C-2 position of the flavanone moiety. This flavanoid was found in the albedo of some citrus fruits. It is responsible for part of the bitter taste of grapefruit fruit. The presence of (2*S*) or (2*R*) naringine depended on grapefruit ripeness measured as fruit diameter. Only one isomer, (2*S*) naringine, was

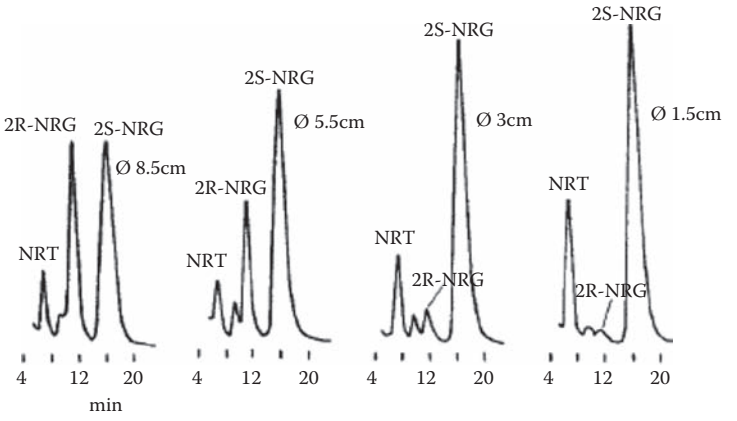

FIGURE 15.5 Chromatogram of naringenine enantiomers (NRG) and narirutin (NRT) from albedo grapefruit as a function of the grapefruit maturity (diameter). (Reprinted from Caccamese, S., Manna, L., and Scivoli, G., *Chirality*, 15, 661–667, 2003. With permission.)

present in immature fruit, while both isomers were present in mature fruit (see Figure 15.5). The investigations were carried out on Chiralcel OD stationary phase [6].

Aglycones of flavanone glycosides (naringenin, eriodictyol, hesperetin, and pinocembrin), which also possess a similar, chiral C-2 center, were investigated using HPLC with two CSPs (Chiralcel OD-H and Chiralpak AS-H). The better chiral discrimination of eriodictyol and hesperidin enantiomers was achieved on AS-H column, while the OD-H column allowed for better separation of naringenin enantiomers. The separation of pinocembrin enantiomers was equivalent on both investigated columns [57].

A crude Japanese drug contained *Auranti nobilis* pericarpium, produced from the peels of *Citrus unshiu* and *Citrus reticulate*. It contains the chiral flavanone 7-*O*-glycosides (hesperidin, narirutin, and neohesperidin). Naturally occurring hesperidin and narirutin have 2*S* configuration. However, in the dry extract from the drugs, the 2*R* epimers were also found. The physiological and biological activities of the epimers were different. Due to this situation, the quantitative analysis of hesperidin and narirutin in medicinal products should be performed. Uchiyama et al. [58] isolated hesperitin, narirutin, and neopesperidin by the HPLC method with a Chiralpak IA column; the chiral selector was amylase (3,5-dimethylphenylcarbamate) immobilized on silica gel. The hesperidin was used as a marker compound for controls of medicines. Investigated peels from both citrus species contain various amounts of 2*R* epimers of hesperidin and narirutin. The amount of narirutin epimer was higher than the amount of hesperidin epimer. In addition, the 2*R* epimer of hesperidin was found in prescriptions for Rikkunshito and Chotosan. It was noticeable that the amount of the 2*R* epimer of hesperidin increased during the decoction process.

Dragocephalum rupestre is a plant that grows in western China. This plant is utilized in traditional Chinese medicine. Due to this an isolation of pure active compounds is necessary. These compounds were dracorupensis A and B. Dracorupensis is novel family of flavanones that possess a fused benzopyran skeleton. They possessed two stereocenters in the molecule (C-2 and C-6), so it was possible to obtain two diastereomeric pairs of enantiomers. An isolation of these compounds from aerial parts of the plant was described by Ron and coworkers [59]. The chiral structure of all four enantiomers of dracorupensis was determined using chiral HPLC (Chiralpack AS-H; Daicel, Tokyo) in combination with on-line CD analysis. All four enantiomers were determined in the plant (see Figure 15.6).

Bamboo grass from the genus *Sasa* shows antimicrobial activity and is used in traditional Asian medicine. It also possess other medicinal activity to cure hypertension, arteriosclerosis, cardiovascular disease, cancer, and so on. Plant active compounds were determined to be triterpenoids, flavone glycosides, and flavonolignans. A hot water extract from *Sasa kurinensis* leaves was investigated

by Hasegava and coworkers [60]. Two new *C*-glycosyl flavones, kurilensin A, and kurilensin B, were isolated. The absolute configuration of the sugar unit was determined on Chiralplack IB. A chromatographic analysis showed that the sugar unit was L-rhamnopyranose in kurilensin A and D-xylopyranose in kurilensin B.

Flavanone-7-*O*-glycosides (naringenin, hesperidin, neohesperidin, narirutin, and eriocitrin) were separated as diastereomers using multidimentional liquid chromatography [61]. The method utilized two various columns—chiral and achiral. A carboxymethylated β-cyclodextrin was the chiral selector in the column. The method was used to compare the flavonoid glycosides contained in fresh hand-squeezed and commercial fruit juices. The diastereomeric ratio of solutes depended on the kind of juice. The orange juice contained (2*S*)-hesperidin and mainly (2*R*)-narirutin, whereas grapefruit juice contained (2*S*)-naringin and (2*R*)-narirutin. A racemate of eriocitrin was present in lemon juice. (2*S*)-hesperidin was also present in this juice. The authors noticed that the ratios of diastereomers depended on juice preparation and storage.

Three methods of investigation were utilized to determine the rate of flavanone glycosides' diastereomerization reaction (stopped-flow multidimensional HPLC, dynamic chiral HPLC, and the classical kinetic method) [62]. A chiral column (Cyclobond I 2000; built from β-cyclodextrins) was used in all methods. The best method of analysis turned out to be the dynamic method. The result suggests that during the processing and storage of orange juice, (2*S*)-hesperidin slowly converted to the (2*R*)-diastereomer. This method was applied in controlling the quality and freshness of citrus juice (see Figure 15.7).

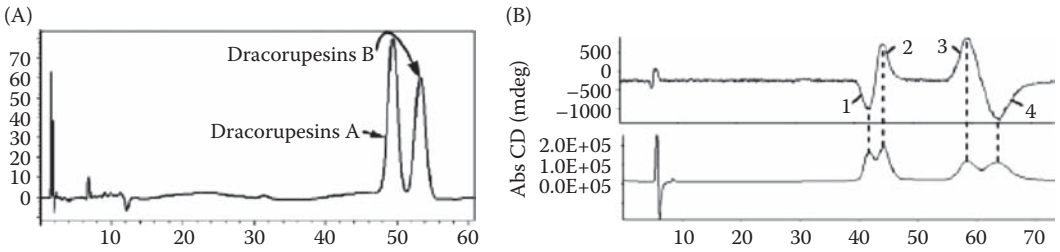

FIGURE 15.6 Chromatograms of dracorupensis separation. (A) HPLC nonchiral separation, column Phenomenex C_{18}; (B) HPLC with Chiralpak AS-H column (upper chromatogram with circular dichroism detection (CDD); lower with UV detection [290 nm]). (Reprinted from Caccamese, S., Manna, L., and Scivoli, G., *Chirality*, 15, 661–667, 2003. With permission.)

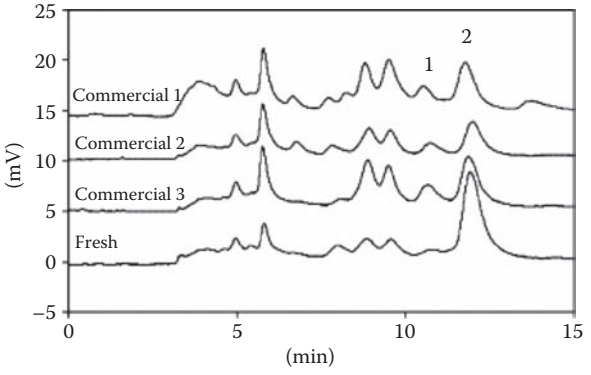

FIGURE 15.7 Chromatograms of the flavanone fraction of freshly squeezed and various commercial orange juices. Chiral column: Cyclobond I 2000. (1) 2*R*-hesperidin; (2) 2*S*-hesperidin. (Reprinted from Asztemborska, M. and Zukowski, J., *J. Chromatogr. A*, 1134, 95–100, 2006. With permission.)

Moghania philippinensis is an herb that grows in southwestern China. It has been used in traditional Chinese medicine to treat osteoscopic pains of rheumatism, arthritis of parturition, leucorrhea, and arthropathy. An ethanol extract also showed cytotoxic activity. Ahn and coworkers [63] isolated five new prenylflavanones and 13 other compounds from *Moghalia* roots. One of the isolated flavanones exhibited the cotton effect. This effect is a characteristic change in optical rotary dispersion and/or circular dichroism in the vicinity of an absorption band of a substance. It is positive if the optical rotation first increases as the wavelength decreases. The compound that showed the cotton effect was separated by HPLC with a chiral column (Chiral CD-Ph; phenylcarbamate β-cyclodextrins). The absolute configuration was determined for both separated enantiomers. The first enantiomer had configuration *R* at C-2, the second *S*.

Flavonoids could be delivered to human organisms as diet components or in herbal medicines. Their level in biofluids might be also investigated. Li and coworkers [64] determined four flavanones (liquiritigenin, naringenin, dihydrowogonin, and dihydrooroxylin A) isolated from human urine after administration of traditional Chinese medicines. Molecules of these compounds still showed optical activity. Due to this property, it is possible to investigate the solute separation on chiral column (Chiralcel OD, Diacel, Tokyo). An improvement in enantioseparation was achieved when acetic acid was added to the mobile phase. It was noticed that *S*-enantiomers of flavanones dominated in urine.

Naringine (4′,5,7-trihydroxyflavanone 7-rhamnoglucoside) is metabolized after consumption to the aglycone bioflavonoid naringenin. Yanez and Davies [65] described the stereoselective HPLC (Chiracel OD-RH, cellulose tris [3,5-dimethylphenylcarbamate] coated on 5 μm silica gel) method for the determination of naringenin. The method was validated and utilized for in vivo kinetic studies of rat and human urine.

Extracts from *Arachis hygopaea*, *Hemizonia increscenes*, *Eriodictyon glutinosum*, and *Thymus vulgaris* were analyzed by chiral HPLC with gradient elution [66]. Cellulose triacetate supported on silica gel diol was used as the CSP. (2*S*)-eriodictyol was found in extracts from peanut hulls (*Arachis hygopaea*) and from *Hemizonia increscens*. (2*S*)-homoeridictyol was produced by *Erodictyon glutinosum*. The (–) enantiomers of naringenin and eriodictyol were found in *Thymus vulgaris* extract. The contents of isomers changed when the extract was heated under racemization conditions (aqueous solution, heated 90 minutes at 90°C). (2*S*)-flavanone enantiomers dominate in extracts. Krause and Galensa analyzed naringenin enantiomers in tomato ketchup extract under polar gradient conditions [66]. Both enantiomers of naringenin were present due to nonenzymatic conversion of naringenin chalcone to flavanone.

Separations of 18 racemic flavanones and diastereomeric flavanone glycosides were carried out on CSPs based on cellulose triacetate (microcrystalline and depolymerized) [67]. One column was commercially available (Chiralcel OA, Daicel), and the second one was specially prepared. Naringenin was among the investigated solutes. Better results for naringenin separation were achieved on the specially prepared column (depolymerized microcrystalline cellulose triacetate coated on Nuclesosil diol stationary phase). Preliminary investigations allowed the determination of the flavanone (pinocembrin, isosakuranetin, naringenin, hesperidin, homoeriodictyol, and eriodictyol) content in tomato peel extract. The analysis was carried out using the HPLC method with gradient elution. All flavanones were determined in both enantiomer forms. The authors explained this phenomenon through racemization that occurred during sample preparation.

15.5 ACIDS

Organic carboxylic acids (CA) are chemical compounds which instead that possess a carboxylic group. There are various acid classes, for example aliphatic, aromatic, cyclic and those with one or more carboxylic groups. These types of chemical compounds are very common in plants. They participate in basic biological metabolism, such as the tricarboxylic acids pathway. They can be found in plants, mostly in fruits, as free or inorganic salts, alkaloid salts, or esters [28].

Only a few of the common known acids are chiral (ascorbic, malic, tartaric acid). Ascorbic acid is one of the vitamins. The chemical name of ascorbic acid is lactone of 2-keto-gulonic acid. Due to the presence of two stereogenic centers, four forms of ascorbic acid (two pairs of enantiomers and two diastereomers) are possible. The biological activity as an anti-ascorbutic compound is exhibited by (+) ascorbic acid, or lactone of 2-keto-L-gulonic acid; (*R*)-3,4-dihydroxy-5-((*S*)-1,2-dihydroxyethyl) furan-2(5*H*)-one. However, all the diastereomers have antioxidant properties [5]. A mixture of L-(+)-ascorbic acid and its epimer, D-(–)-isoascorbic acid was separated on a DL-phenylpropanol amine-coated column using multimodal chromatography. The method was used to analyze L-(+) ascorbic acid in orange juice [68].

Malic acid exists in nature mostly as the L-(–) isomer but sometimes could be found as the D-(+) form. It belongs to the most popular acids. Malic acid enantiomers were separated by the HPLC method with a ligand-exchange chiral mobile phase (*N*,*N*-dimethyl-L-valine and Cu^{2+}) in adulterated apple juice [69]. This method was also utilized to determine the D-isomers in apple, pear, and grape juices [70]. The enantiomeric purity of malic acid in apple juices was determined using multibeam circular dichroism detection (CDD) (see Figure 15.8.) [7].

Mandelic acid belongs to the aromatic hydroxyl acids. It appears in bitter almonds or in cyanogenic glycosides. Analysis of mandelic acid using transfer complexes (Pirkle-type CSPs with zeolites as support; benzene-L-leucine or *N*-[3,5-dinitrobenzoyl]-L-leucine) with Raman spectroscopy was described by Horvath et al. [71]. The authors used Raman spectroscopy to investigate interactions between the stationary phase and analyte. The ability to form hydrogen bonds in the stationary phase increased as its π-acceptor character decreased. Mandelic acid and its derivative enantiomers were separated on ligand-exchange stationary phases synthesized from L-amino acids [72]. Copper (II) ions were used as a complexing agent in all phases. The best separation was achieved using a stationary phase built from L-hydroxyproline. The (+) enantiomer eluted before the (–) enantiomer.

A tropic acid also belongs to this group of acids. It is a constituent of tropane alkaloids. [28] Racemic mandelic and tropic acids and their various relative compounds were separated directly on a *t*-butylbenzoylated tartaric acid-based CSP (Kromasil-CHI-TBB) both on the analytical and the preparative scale [73]. The authors investigated interactions between the chiral selector in the

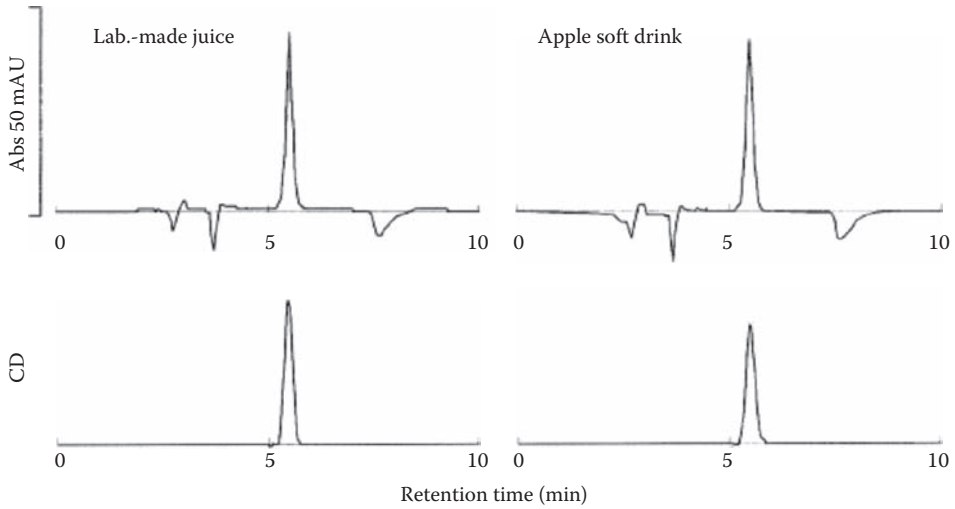

FIGURE 15.8 Analysis of malic acid in apple juice: lab-made juice and apple soft drink. Upper part, UV detection; lower part, circular dichroism detection (CDD). (Reprinted from Yamamoto, A., Akiba, N., Kodama, S., Matsunaga, A., Kato, K., and Nakazawa, H., *J. Chromatogr. A*, 928, 139–144, 2001. With permission.)

stationary phase and the separated solutes. The dominant interactions that occurred were hydrogen bonding. Also, the influence of solute structure and the composition of the mobile phase on enantioseparation was investigated.

Acids or their derivates could be contact poisons for insects and coldblooded vertebrates in some plants. *Tanacetum cinerariaefolium* (*Pyrethrum daisy*) is one of them. The derivatives of pyrethroic acid (*cis*/*trans*-chrysanthemic acid; *cis*/*trans*-chrysanthemum dicarboxylic acid, *cis*/*trans*-permethrinic acid and fenvaleric acid) were found in extracts of this plant. All these acids were separated into enantiomers and diastereomers using the HPLC method with an enantioselective anion-exchange stationary phase (carbamylated cinchoma alkaloid-derived CSP) both in reversed-phase and normal-phase mode [74]. Better resolution of chrysanthemic acid was obtained for reversed-phase conditions.

Organic acids could also be endogenic plant growth regulators. They occurred in plant tissues upon stress treatment. These type of compounds were jasmonic acid and cucurbic acid. Both acids could form a diastereomeric pair with amino acids (peptide-like conjugates). This type of conjugates also occurred in plants. The resolution of the diastereomeric pair of jasmonic and cucurbic acid with some amino acids (isoleucine, leucinol, tyramine) was achieved on both nonchiral (RP-18) as well as chiral (Chiralpack AS; Nucleodex β-PM; permethylated–β-cyclodextrin) stationary phases [75]. Better separation was obtained for conjugates that possess the polar carboxyl or hydroxyl group in the amino acid part of the conjugate. The enantiomeric pair of conjugated jasmonic acid with tyramine or isoleucine was separated only on the chiral column. The order of elution of solutes depended on the chirality of the jasmonide part of the conjugated compounds and was as follows: enantiomer with the configuration RR earlier, next SS. Chiralpack AS stationary phase exhibited better separation of peptide-like conjugates of both investigated acids, which indicated the possibility of utilizing this type of column also in analysis of jasmonic and cucurbic acid from natural sources.

Other plant growth regulators are 3-(3-indolyl)-butyric acid, abscisic acid, and other compounds with an indole ring. Enantiomers of these type of solutes were separated on macrocyclic antibiotic CSPs (vancomycin, teicoplanin, and ristocetin A) using HPLC and capillary electrophoresis (CE) method [76]. The enantioselective resolution is similar on all these types of CSP columns. However, differences in secondary stereospecific interactions between stationary phases and solutes caused improvement of the enantioseparation if the CSP was changed.

Chiral fatty acids are present in various Asian lichen species. The enantiomers of methyl naphtoyl derivatives of 15-hydroxyloctadecanoic acid were separated with good resolution on an (*R*)-(–)-*N*-(3,5-dinitrobenzoyl)-α-phenylglycine (DNBPG) CSP [77]. The oxirane ring of *cis*-9,10-epoxystearic acid could be transformed to *threo*-9,10-dihydroxystearic acid by epoxide hydrolase from soybean [78]. This reaction is stereospecific. The enzyme prefers *cis*-9*R*,10*S*-epoxystearic acid. The high enantioselectivity of the epoxide hydrolase depends on a free carboxylic acid functionality on the substrate, which probably influences its positioning within the active site. The course of this reaction was investigated by chiral liquid chromatography with Chiralcel OB column (cellulose tribenzoate coated on 10 μm silica gel).

The enzymes from the marine green alga *Ulva conglobata* transformed linoleic and linolenic acid into corresponding (*R*)-9-hydroperoxy-(10*E*,12*Z*)-10,12-octadecanoic acid ((R)-HPODE) and (*R*)-9-hydroperoxy-(10*E*,12*Z*,15*Z*)-10,12,15-octadecatrienoic acid ((R)-HPOTrE). The absolute configuration of this reaction's products—hydroxy methyl esters—was determined using HPLC with a Chiralcel OD-H column [79].

The mixtures of enantiomers of 2-hydroxy acids and amino acids in foodstuffs were separated using columns coated with *N*,*N*-dioctyl-L-alanine [80]. The resolution of enantiomers was achieved by forming the transition complexes with a chiral selector possessing the copper (II) ion. The order of enantiomer elution depends on the position of the hydroxyl group. For 2-hydroxy acids, which possess the hydroxyl group bonded in α-position to the carboxyl group, the order was as follows: D-isomer, then L-isomer (e.g., mandelic acid). When the position of the hydroxyl group was β, as in tartaric and malic acid, the order was L-enantiomer before D-enantiomer.

Theanine is a nonproteinic amino acid found in tea leaves. Native and derivatized theanine enantiomers were separated on the Chirobiotic T CSP [81]. The HPLC method was coupled with atmospheric pressure ionization mass spectrometry (API-MS) detection. The method was utilized to evaluated the content of L-threonine in commercially available samples. Some samples contained the D-enantiomer as well as the L-enantiomer.

15.6 OTHER CHIRAL NATURAL COMPOUNDS

There is a variety of chiral natural products. In the following, the analysis of some of them by the chromatographic chiral mode is presented. The atropoisomers of austrocolorins A_1 and B_1 (10,10'–couplet dihydroanthracenones) were isolated from the Australian plants *Dermocybe* sp. Next, the structures of isolated compounds were confirmed by ^{13}C NMR. The absolute configuration at the stereogenic center in austrocolorin A_1 was confirmed chromatographically after chemical reduction to (*S*)-torosachrysone-8-*O*-methyl ether and comparison with an authentic sample of (*S*)-torosachrysone-8-*O*-methyl ether. This procedure was carried out using Chiralpak AD. The mobile phase was pure ethanol. The retention time of the (*S*)-enantiomer was 10.1 min, whereas the retention time of the (*R*)-enantiomer was 28.7 min [82].

The enantiomers, anomers, and structural isomers of some biologically relevant monosaccharides were investigated by a one-step chiral HPLC method with a Chiralpak AD-H column [83]. The method allows the determination of solutes such as glucose, fructose, arabinose, and others, including in biological compounds. The method was validated. The good results achieved allow on-line separation of nine compounds (see Figure 15.9). Another sugar, cladionol A (polyketide glycoside), was isolated from *Gliocladium* sp. and then analyzed by Yuu et al. using the chiral HPLC method to confirm the presence of β-mannopyranoside units in the D-form [84].

Phosphatidylglycerols (PGs) from cabbage leaves, soybeans, and *Escherichia coli* were investigated as bis-(3,5-dinitrophenylurethanes) on two columns with opposite configurations (*R*)(+)- and *S*(–)-1-(1-naphthyl) ethylamine as chiral selectors. Lipids from cabbage and soybeans consisted of only one isomer (*R*,*S*-1,2-diacyl-sn-glycero-3-phosphoro-1'-sn-glycerols) despite the presence of nonspecific phospholipase D. Lipids from *E. coli* contain also a significant amount of the (*R*,*R*)-isomer [85]. The algal lipids after their conversion to PGs were isolated from various green algae

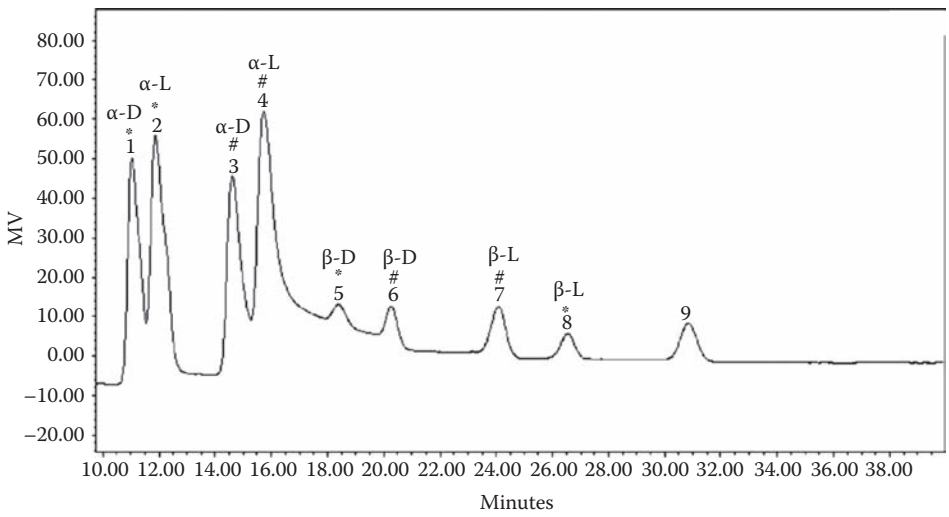

FIGURE 15.9 Chiral analysis of sugars mixture. (1, 2) α-L-; D-mannopyranose; (3,4) α-L, D-lyxopyranose; (5) β-D-mannopyranose; (6) β-D-lyxopyranose; (7) β-L-lyxopyranose; (8) β-L-mannopyranose; (9) furanose. (Reprinted from Lopes, J.F. and Gaspar, E.M.S.M., *J. Chromatogr. A*, 1188, 34–42, 2008. With permission.)

by preparative thin-layer chromatography and, after conversion into their bis (3,5-dinitrophenylurethane) derivatives, were separated on (*R*) and (*S*)-1(1-naphythyl)ethylamine CSPs. In a similar way, PGs from other plants such as spinach, radish, parsley, and green tea were also examined and compared with the algal ones. All investigated PGs consisted of 1,2-diacyl-sn-glycero-3-phosphoro-1'-sn-glycerol (*R*, *S* configuration).

The leaves of *Fraxinus americana* contain oleuropein-type secoiridoid glycosides. Takenaka et al. isolated several new compounds of this type [86]. Their spectra were determined by ^{13}H NMR. Stereochemistry at C-2'' atom of solutes was determined using liquid chromatography with Chiralcel OB-H as the stationary phase. The comparison of the retention times with proper standard solute confirmed the presence of O-function at C-2" atom and the investigated compounds were (2'' *R*)—2''-hydroxyoleuropein and (2'' *S*)—2''-hydroxyoleuropein.

Xanthophylls such as 3, 3'-zeaxanthin are phytonutrients widely distributed in orange-colored fruits and vegetables (corn, wolfberries, orange pepper, sea buckthorn) and are also present (as a minor component) in green leafy vegetables (spinach, lettuce). In plants only one stereoisomer (3*R*,3'*R*)-zeaxanthin was found. Schlatterer et al. [87] described a new method of determination, diastereomeric dilution assay (DIDA), of this zeaxanthin as a diastereomer after mixing with meso-zeaxanthin. The solute was determined by HPLC with amylose tris (3,5-dimethylphenylcarbamate) as the chiral selector. This method was validated. Concentrations of (3*R*,3'*R*)-zeaxanthin in plant-derived food obtained by external calibration and the DIDA method were compared and showed that both methods (external calibration and DIDA) may be used for determination of (3*R*,3'*R*)-zeaxanthin.

The bioactive compounds that inhibit hyaluronidaze are present in peanuts' skin fraction. Among them, there were A-type prothocyanidins dimmers. Lou's group isolated three new ones: epicatechin- (2β→O→7, 4β→6)-catechin; epicatehin-(2β→O, 4β→60-*ent*-catechin; and epicatechin-(2β→O→7, 4β→5)-*ent*-epicatechin [88]. After conversion of the prothocyanidins to monomers by reductive cleavages, the solutes were investigated on a Chiralcel OD column.

Two new procyanidin trimers derivatives of galloyl epicatechine were isolated from *Byrsonina crassifolia* bark, a medicinal plant of the Mexican Indians [89]. The enantiomeric purity of isolated solutes was checked by HPLC with a cyclodextrin chiral column and compared to authentic material of known configuration. Some obtained compounds were pure 2*S*-enantiomers, whereas some were a mixture of isomers.

Aerial parts of *Stemona parviflora* Wright contain biologically active compounds—dimeric phenylethyl benzoquinnes (parvistemins). Four of them, parvistemins A–D, were isolated by Yang's group [90]. Due to restricted rotation about the central biaryl axis, they are chiral. The analytical resolution of these compounds was done using the chiral HPLC method (Chiralcel OD-H as chiral selector) coupling with circular dichroism (LC-CD). The method of analysis was also connected with quantum chemical CD-calculations using the OM2 Hamiltonian and a TDDFT approach. The investigated solutes appeared racemic in nature, which is a well-known phenomenon among the axially chiral natural products. Determination of the absolute configuration of parvistemin A enantiomers is shown in Figure 15.10.

The fractions from roots of *Bulbine frutescens* were investigated, and sulfated natural products knipholone and their sulfated free analogues were isolated [91]. The new compounds possess the biaryl axis and are therefore optically active. The isomers of *O*-demethylknipholone were separated on a Chiral OD-H column, and it appears that isolated compounds were racemic mixture or racemate. The obtained separations make possible to semipreparative separations. The second group of isolated compounds was sulfated solutes, which had highly polar nature, so they could not be separated using the chiral HPLC mode. However, after the acidic hydrolysis of respective sulfates, resolution on the same chiral column was possible. Not all compounds were enantiomerically pure.

Plants infected by pathogens produce secondary metabolites called phytoalexins. One of the phytoalexins is 3-hydroxy-α-calacorene. It was isolated from extracts of cold-shocked cotton, kenaf, and *Hetoretheca inuloides* (Malvaceae family). In addition, the *Hetoretheca inuloides* produced the

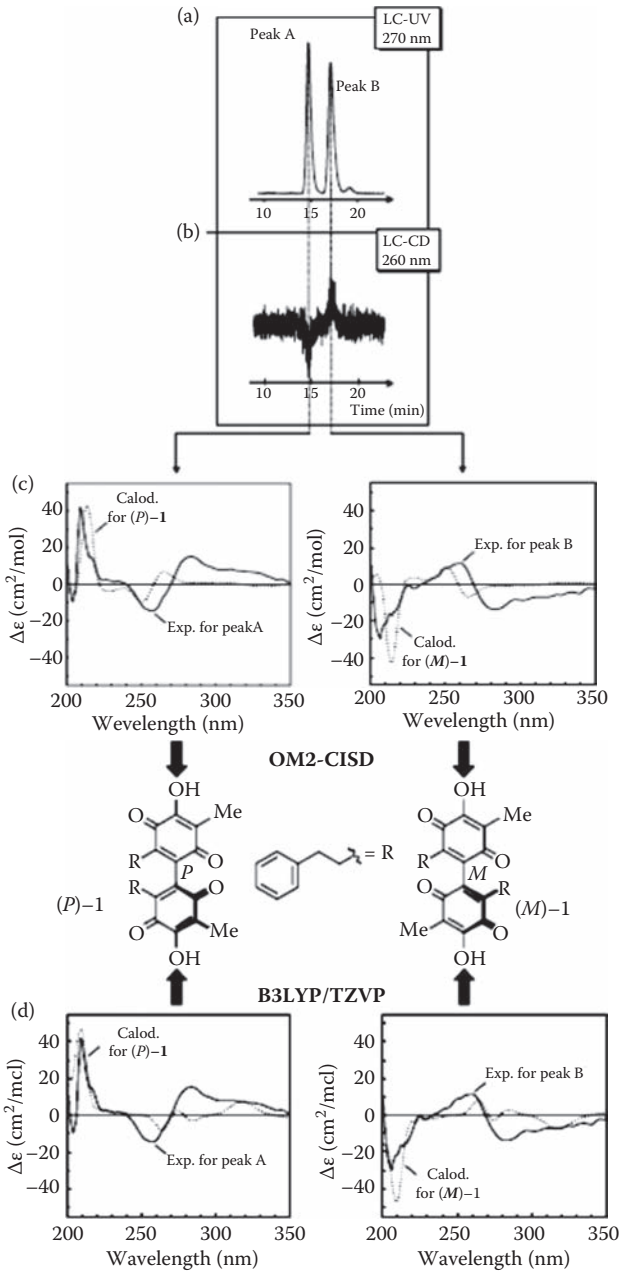

FIGURE 15.10 Determination of the absolute configuration of enantiomers of parvistemin A. (Reprinted from Yang, X., Gulder, T.A.M., Reichert, M., Tang, C., Ke, C., and Bringmann, G., *Tetrahedron*, 63, 4688–4694, 2007. With permission.)

3-hydroxy-α-calacorene but (–) isomer. During the chiral chromatographic analysis, with (*R*)-*N*-(3,5-dinitrobenzoyl)-phanylglycine as the CSP, it appeared that 3-hydroxy-α-calacorenes isolated from cotton and kenaf seedings have the same absolute configuration, and this configuration was opposite to that from *Hetoretheca inuloides* [92].

Isoshinanolones are compounds with a tetralone skeleton. They are widespread in various plant families. They may be produced when plants are stressed. Enantiomers are formed due to chemical

structure (naphthoquinone derivatives). The *cis*- and *trans*-isoshinanolones were isolated from the African plant *Dioncophyllum thollonii*. Their absolute configuration was determined by the chromatographic method after the ruthenium catalyzed oxidative degradation. The chromatographic HPLC analysis was carried out on the chiral column Chiralcel OD-H. It appeared that both *cis*- and *trans*- isomers isolated from the plant were enantiomerically pure [93]. The on-line chiroptical stereoanalysis by HPLC with a CSP coupled with CD spectroscopy permitted the determination of the absolute structure of all four stereoisomeric forms of isoshinanolones in one run [94].

Plants are one of the sources of peptides. The cyclic peptides show a wide range of biological activity. The new cyclic peptides (cyclosquamosins A–G) were isolated from the seeds of *Annona squamosa* [95]. The sequence of amino acids was elucidated after hydrolysis. The chiral HPLC analysis (Sumichiral OA-5000 column) shows that all amino acids were in L conformation.

Gossypol (2,2'-bis-(formyl-1,6,7-trihydroxy-5-isopropyl-2-methylnaphthalene) is a polyphenol derived from the cotton plant (genus *Gossypum*, family *Malvaceae*). Gossypol is chiral due to steric hindrance to rotation about the internaphthyl bond. It permeates cells and acts as an inhibitor of several dehydrogenase enzymes. The procedure of isolating gossypol from plants affords racemic gossypol as a 1:1 complex with acetic acid. The ratio of gossypol enantiomers depends on the kind of plant [96]. It was determined that the (–) enantiomer of gossypol is pharmacologically more active than the (+) isomer. Separation of gossypol enantiomers on an analytical and preparative scale was achieved on a cellulose *tris*-(3,5-dimethylphenylcarbamate) CSP [97,98].

Lignans are compounds which possess carbon skeletons formed from two phenylpropane units. They are connected with lignin biosynthesis [28]. They could be found either in enantiomeric excess or in enantiomerically pure (+) or (–) form. Gymnosperm *Thuja* species (*Cupressaceae*) accumulate characteristic lignans with a 3,5-dimethoxy-4-hydroxyphenyl (syringyl) group. Five lignans, (8*R*, 8'*R*)-(–)-matairesinol, (8*R*, 8 '*R*)-(–)-thujaplicatin methyl ether, (8*S*,8'*S*)-(–)-wikstromol, 8-hydroxythujaplicatin methyl ether, and epi-pinoresinol, were isolated from *Thuja occidentalis* branch xylem, besides the previously identified (8*R*, 8'*R*)-(–)-4-*O*-demethylyatein. Chiral HPLC (Chiralcel OD) analyses of matairesinol, thujaplicatin methyl ether, wikstromol, and 4-*O*-demethylyatein indicated that these compounds were optically pure [99].

Lignans from *Forsythia intermedia* were investigated by a reversed-phase HPLC method with UV/laser polarimetric detection [100]. The method was developed for the direct determination of lignan chirality and enantiomeric composition. It was suitable also for analyses of stereoselective monolignol radical–radical coupling assay mixtures containing the (+)-pinoresinol forming dirigent protein, and of lignan chirality in crude plant extracts (by utilizing the Chiralcel OD column). Eudesmine (nonphenolic, furofuran lignans) and pinoresinol were isolated from *Magnolia kobus* DC var. *Borealis sarg*. Utilizing HPLC and a Chiralcel OD column, it was demonstrated that pinoresionol was present as a mixture of (–) and (+) enantiomers. The (+) enantiomer predominated (77.1% enantiomeric excess). Eudesmine was isolated only as the (+) antipode [101].

15.7 SUMMARY

Plants generally produce one enantiomer. However, during maturation, storage, or processing racemization takes place and the amount of enantiomers can be changed. Enantiomers produce various physiological, pharmacological, and toxicological effects. Due to these effects, the determination of absolute configuration seems to be necessary. NMR, MS, CD, optical rotation, and X-ray crystallography have been used to solve this problem. However, chiral HPLC combined with these classical methods may be the method of choice for determination of absolute configuration. Advantages of this method are the short analysis time, wide availability of chiral columns, and effectiveness. The establishing of the absolute configuration of unknown compounds can be done in one step from the comparison of the retention time of the investigated solute with the reference enantiomer whose absolute configuration is well known, but further confirmation of the absolute configuration with the help of MS or CD is necessary. A combination of analyzed solutes and chosen CSPs is presented in Table 15.2.

TABLE 15.2
Separation of Various Natural Chiral Plant Compounds on Various Chiral Stationary Phases

Solute	Chiral Stationary Phase	References
	Alkaloids	
Erbutamine	Chiralpak AD	[37]
Aristoyagonine	Chiralcel OD-H;	[38]
N-*p*-methyl-1-α-dihydroaristoyagonine;	Chiralpak IB	
4',5'-dimethoxyl-1-α-dihydroxyaristoyagonine	2R,3R-β-GEM	
	Kromasil CHI-DMB	
DL-Tetrahydropalmitine, DL-tetrahydroberberine	Cellulose-3,5-dimethylphenylcarbamate	[39]
Synephrine	Sumichiral OA-6000	[40]
Synephrine	β-CD	[41]
	Chiral-CBH	[42]
	Sumichiral OA, Chiradex, Chiral-CBH	[43]
	Chiral-CBH	[44]
Dracocephins A, B, C, and D	Chiralpak AS-H	[45]
Tacamonine, vindeburnol	Chiralpak AD	[46]
	Chiralcel OD	
9-Methoxymitralactonine	Chiralcel OD	[47]
Tobacco alkaloids	Chiralcel OD-H	[48]
Dioncophylline E	Chiralcel OD	[49]
Dioncophylleine A	Chiralcel OD-H	[50]
Lisuride, terquride, meluol, nicergoline	Vancomycin; teicoplanin	[51]
Atropine, scopolamine	Chiracel OD	[52]
Atropine	α_1-Acid glycoprotein	[53]
Sugar unit from cephalezomine	Chiralpak OP (+)	[54]
	Flavanones	
Derivatives of maesospin	Chiralcel OD	[56]
Naringin	Chiralcel OD	[6]
Naringenin, eriodicyol, hesperitin, pinocembrin	Chiralcel OD-H	[57]
	Chiralpak AS-H	
Hesperidin, narirutin, neohesperitin	Chiralpak IA	[58]
Dracorupensis A, dracorupensis B	Chiralpak AS-H	[59]
Kurilensin A, kurilensin B	Chiralpak IB	[60]
Naringenin, hesperidin, neohesperidin, narirutin, eriocitrin	Carboxymethylated β-cyclodextrins	[61]
Flavanone glycosides	Cyclobond I 2000	[62]
Prenylflavanones	Chiral CD-Ph	[63]
Liquiritigenin, naringenin, dihydrowogonin, dihydrooroxylin A	Chiralcel OD	[64]
Naringenin	Chiralcel OD-RH	[65]
Homoeridictyol, naringenin, eriodictyol	Cellulose triacetate	[66]
18 flavanones and flavanone glycosides	Cellulose triacetate	[67]
	Acids	
Ascorbic and isoascorbic acid	DL-Phenylpropanol amine	[68]
Malic acid	(*N*,*N*-dimethyl-L-valine and Cu^{2+}), LEC	[69]
Malic acid	(*N*,*N*-dimethyl-L-valine and Cu^{2+}), LEC	[70]

TABLE 15.2 (CONTINUED)
Separation of Various Natural Chiral Plant Compounds on Various Chiral Stationary Phases

Solute	Chiral Stationary Phase	References
Mandelic acid	Benzene–L-leucine (LeuB) *N*-(3,5-dinitrobenzoyl)-L-leucine (LeuDNB) *N*-(3,5-dinitrobenzoyl)-L-phenylalanine (PheDNB)	[71]
Mandelic acid	L-Amino acids L-Hydroxyproline	[72]
Mandelic and tropic acid	*t*-Butylbenzoated tartaric acid (Kromasil–CHI-TBB)	[73]
Pyrethroic acid, *cis*/*trans* chrysanthemic acids, *cis*/*trans* chrysanthemum dicarboxylic acid, permethrinic acid, fenvaleric acid	Carbamylated cinchoma alkaloid-derived CSP	[74]
Jasmonic acid, cucurbic acid	Chiralpak AS Nucleodex β-PM	[75]
3-(3-indolyl)-butyric acid, abscisic acid	Vancomycin Ristocetin A	[76]
Methyl naphthoyl derivatives of 15-hydroxyoctadecanoid acid	DNBPG	[77]
cis-9,10-Epoxystearic acid; *threo*-9,10-dihydroxystearic acid	Chiralcel OB	[78]
(*R*)-9-hydroperoxy-(10*E*, 12*Z*)-10,12, octadecanoic acid; (*R*)-9-hydroxyperoxy-(10*E*, 12*Z*, 15*Z*)10,12,15-octadecatrienoic acid as hydroxylmethyl esters	Chiralcel OD-H	[79]
2-Hydroxy acids	*N*,*N*-dioctyl-L-alanine	[80]
Theanine	Chirobiotic T	[81]
Other Compounds		
Austrocolorins A_1, B_1 as torosachrysome-8-*O*-methyl ethers	Chiralpak AD	[82]
Monosaccharides	Chiralpak AD-H	[83]
Cladionol A	Chiralpak OP (+)	[84]
Phosphatidylglycerols as bis-(3,5-dinitrophenylurethans)	R-(+)-1-(1-naphthyl) ethylamine S(–)-1-(1-naphthyl) ethylamine	[85]
Oleuropein-type secoiridoid glycosides	Chiralcel OB-H	[86]
(3*R*,3'*R*) zeaxanthin	Amylose tris (3,5-dimethylphenylcarbamate)	[87]
Protocyanidin dimers	Chiralcel OD	[88]
Galloyl epicatechine derivatives	Cyclodextrin	[89]
Parvistemins	Chiralcel OD-H	[90]
Knipholone; *O*-demethylknipholone	Chiralcel OD-H	[91]
Phytoalexins (3-hydroxy-α-calacorene)	DNBPG	[92]
Isoshinanolones	Chiralcel OD-H	[93,94]
Cyclosquarnosins A–G	Sumichiral OA-5000	[95]
Gossypol	Cellulose tris-(3,5-dimethylphenylcarbamate)	[96–98]
Lignans, matairesinol, thujaplicatin methyl ether, wikstromol, 4-*O*-demethylatein	Chiralcel OD	[99]
Pinoresinol	Chiralcel OD	[100]
Eudesmine, pinoresinol	Chiralcel OD	[101]

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Part II

Primary Metabolites

16 HPLC of Carbohydrates

Angelika Koch, Simla Basar, and Rita Richter

CONTENTS

16.1 INTRODUCTION

Carbohydrates are the most abundant of the groups of biomolecules, which also include proteins, lipids, and nucleic acids. In plants, carbohydrates are formed as a result of photosynthesis. They play a pivotal role in organisms and systems in biology, physiology, and medicine, such as controlling the processes of the immune system, fertilization, pathogenesis, blood clotting, growth, and development. Carbohydrates serve as an energy source for metabolism and as a form of storage (e.g., starch in plants, glycogen in animals and humans), and they provide structural components for stabilization (e.g., cellulose in plants, chitin in animals).

Monosaccharides are single carbohydrate units. They are classified according to three different characteristics: the number of carbon atoms they contain, the placement and function of the carbonyl group, and their chiral center. A straight-chain monosaccharide will form heterocyclic rings by the intramolecular nucleophilic addition of a hydroxyl group to the carbonyl function. This carbonyl function originates from an aldehyde or a ketone group, building hemiacetals and hemiketals,

respectively. The aldehyde group results in aldoses, and the ketone group in ketoses, respectively (Figure 16.1).

Rings with six carbon atoms are called pyranose and those with five atoms are called furanose forms, and they exist in equilibrium with their straight-chain form (see Section 16.1.1).

Monosaccharide units (e.g., an aldohexose $C_6H_{12}O_6$) are used to build various kinds of oligosaccharides (2–10 monosaccharide units) and polysaccharides (from 11 to hundreds of monosaccharide units). For more detailed accounts of carbohydrates, see References [1–3].

16.1.1 Characteristics and Properties

In sugar chemistry, an *anomer* is a special type of epimer. It is a stereoisomer (diastereomer, more exactly) of a saccharide (in the cyclic form) that differs only in its configuration at the hemiacetal (or hemiketal) carbon, also called the *anomeric carbon*. During the conversion from straight-chain form to cyclic form, the anomeric carbon atom containing the carbonyl oxygen becomes a chiral center with two possible configurations: The oxygen atom may take a position either above or below the plane of the ring.

In the α anomer, the –OH substituent on the anomeric carbon rests on the *trans* (opposite) side in the *axial* position of the ring like the CH_2OH substituent, while in the β anomer, the CH_2OH substituent and the anomeric hydroxyl are on the *cis* (same) side in the *equatorial* position of the plane of the ring. The two different anomers are two distinct chemical structures, and thus have different

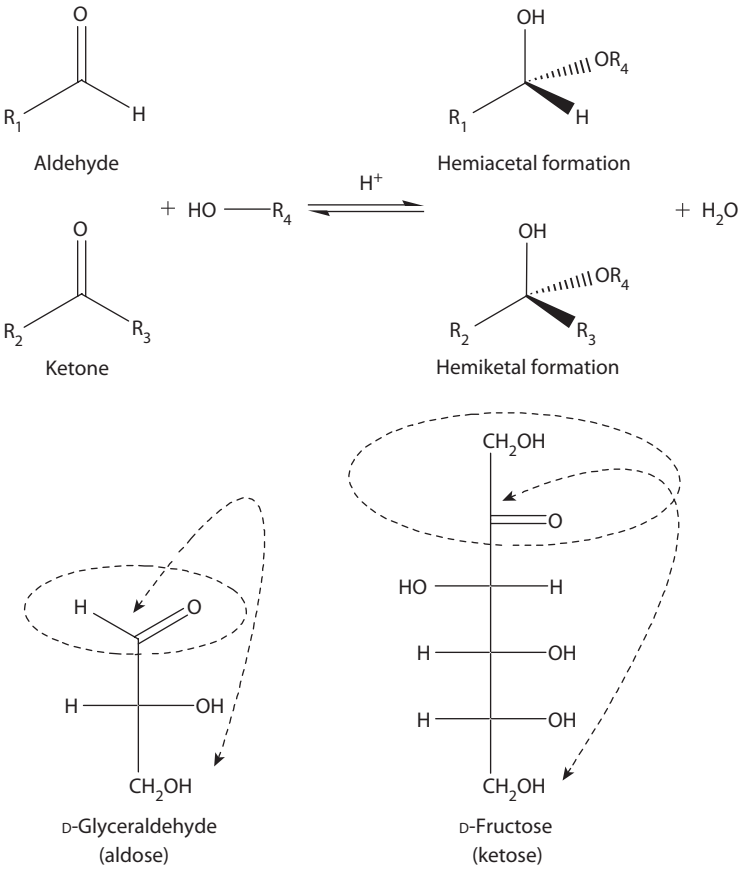


FIGURE 16.1 Carbonyl function.

physical and chemical properties, notably optical rotation. When α-D-(+)-glucose is dissolved in water, the specific rotation of the sample decreases from a value of +112° to a value of +53°, corresponding to an equilibrium mixture of α-D-(+)-glucose and β-D-(+)-glucose; (pure) β-D-glucose has an optical rotation of +19°.

Mutarotation is the term given to the change in the specific rotation of a cyclic monosaccharide as it reaches an equilibrium between its α and β anomeric forms of pyranoses and—to a minor extent—of furanoses, too (Figure 16.2). Though the cyclic forms are usually favored, monosaccharides in aqueous solutions are always in equilibrium with their straight-chain forms. This equilibrium is established as the hemiacetal bond between C1 (the only carbon bound to two oxygens) and C5 is cleaved (forming the straight-chain compound) and reformed (forming the cyclic compound) (Figure 16.2).

16.1.2 Nomenclature

Monosaccharides with three carbon atoms are called trioses, those with four are called tetroses, those with five are called pentoses, those with six are hexoses, and so on. If the carbonyl group is an aldehyde, the monosaccharide is an aldose; if the carbonyl group is a ketone, the monosaccharide

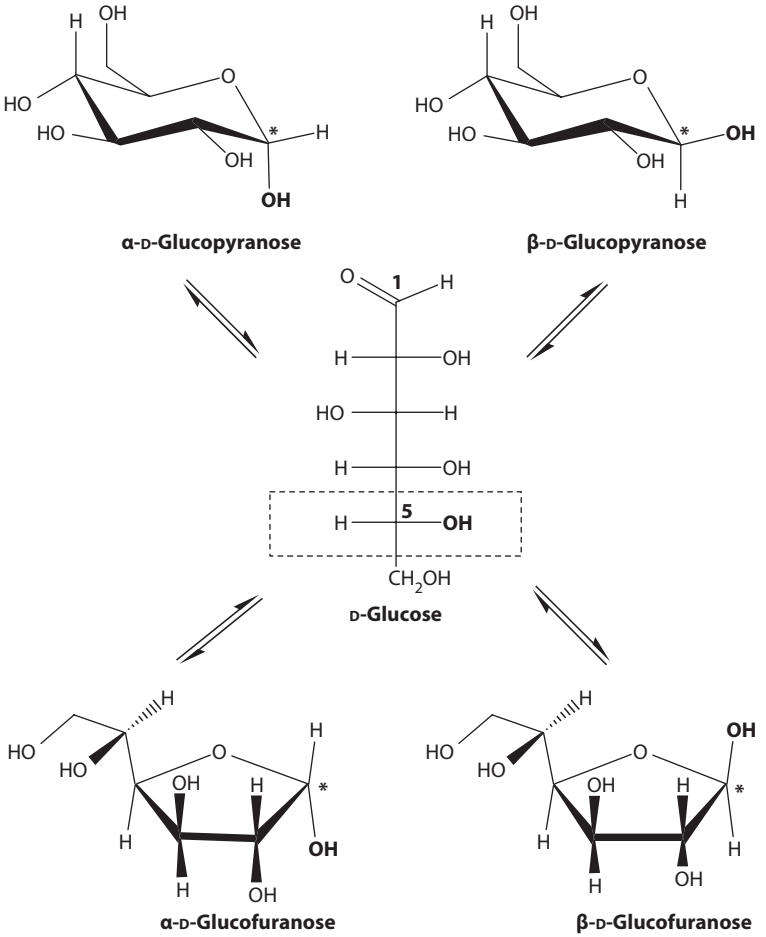


FIGURE 16.2 Mutation of D-glucose in its straight-chain form leads to cyclic α- and β-pyranoses and α- and β-furanoses as well. The anomeric center of carbon C5 is marked by an asterisk.

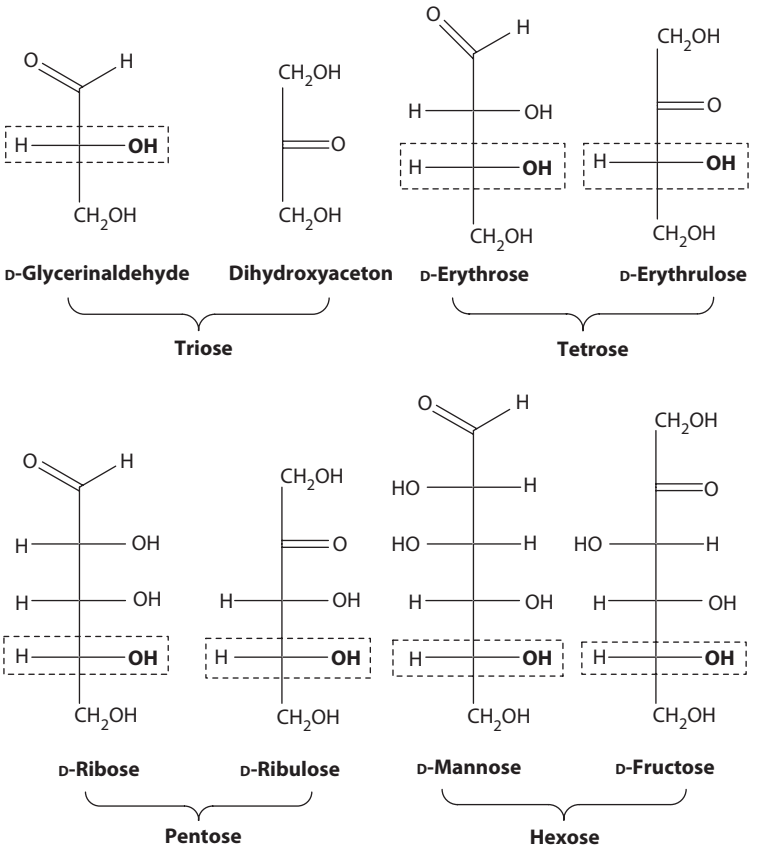


FIGURE 16.3 Examples of trioses, tetroses, pentoses, and hexoses as aldoses or ketoses. The right or left position of the bold hydroxyl group in the boxes assigns them to the D- or L-configuration.

is a ketose. These two systems of classification are often combined. For example, glucose is an aldohexose (a six-carbon aldehyde), ribose is an aldopentose (a five-carbon aldehyde), and fructose is a ketohexose (a six-carbon ketone) (Figure 16.3). Trivial names for sugars are commonly used. The structural complexity of saccharides is due to the fact that two sugars can be linked together, through an ether linkage, in a number of different ways. This ether linkage can have either an α- or a β-configuration, due to the stereochemistry of simple sugars.

Carbons with hydroxy groups are asymmetric, making them stereocenters with two possible configurations each. The D- or L- prefix of Fischer's projection—relative to one enantiomer of glyceraldehyde—is still used, where a configurational reference group on the left side is termed L, for *levo*, and on the right side, D, for *dextro*. The absolute configurations of carbohydrates—based on the Cahn–Ingold–Prelog system—in terms of *R* (*rectus*) or *S* (*sinister*) are determined according to the direction of the classified substituents. They are, however, not commonly used (Figure 16.4). Because of their asymmetry, a number of isomers may exist for any given monosaccharide formula.

16.1.3 Monosaccharides and Their Derivatives

In plants, monosaccharides (and oligosaccharides, too) are rarely found in the free state but often as a component of plant glycosides. They are derived from the respective reducing pyranoses and furanoses (Figure 16.5). Monosaccharide units are modified by deoxygenation (digitoxose, fucose,

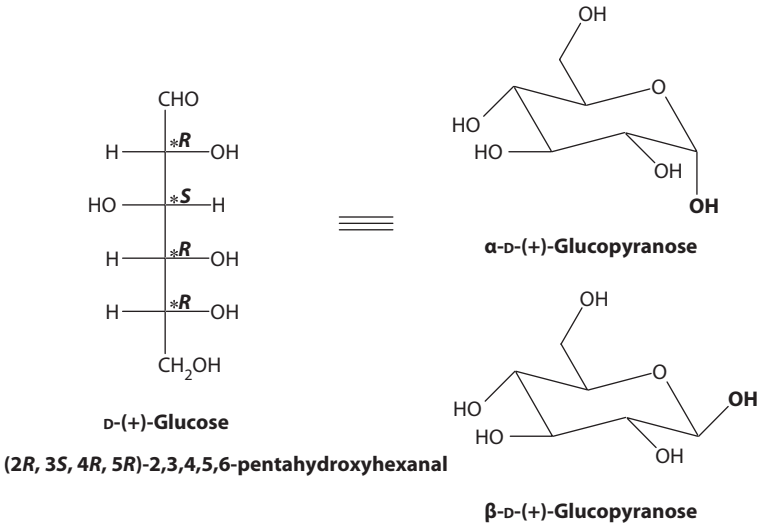


FIGURE 16.4 Configuration of D-glucose drawn by Fischer projection (described according to the Cahn–Ingold–Prelog rules) and the corresponding chair conformations.

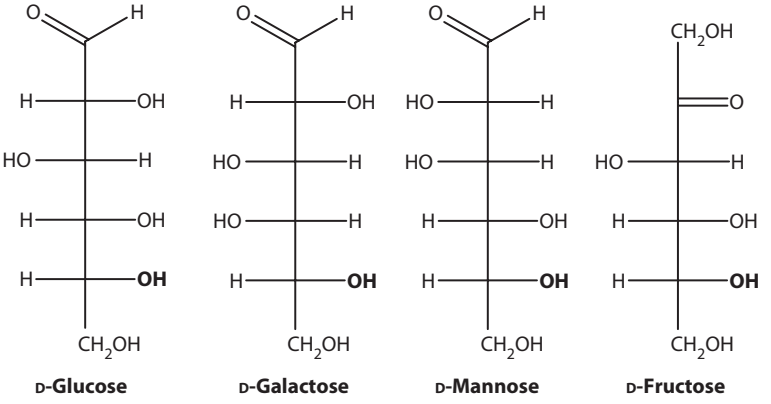


FIGURE 16.5 Monosaccharide units. The bold hydroxyl group assigns them to the D-configuration.

rhamnose), amino groups (glucosamin), methylation (cymarose), reduction (alditols, sugar alcohols, e.g., mannitol), and oxidation (sugar acids, e.g., glucuronic acid); see Figure 16.6.

16.1.4 Disaccharides

Two joined monosaccharides are called a disaccharide and represent the smallest oligosaccharides. They are composed of two monosaccharide units bound together by a covalent bond known as a glycosidic linkage formed via a dehydration reaction, resulting in the loss of a hydrogen atom from one monosaccharide and a hydroxyl group from the other. The formula of an unmodified disaccharides is $C_{12}H_{22}O_{11}$. Although there are numerous kinds of disaccharides, a handful of disaccharides are particularly notable: saccharose (equal to sucrose), maltose, cellobiose, lactose, and trehalose (Figure 16.7). Some of them belong to the reducing carbohydrates (maltose family), where both hemiacetals are linked together, forming a mixed acetal, and some to the nonreducing carbohydrates

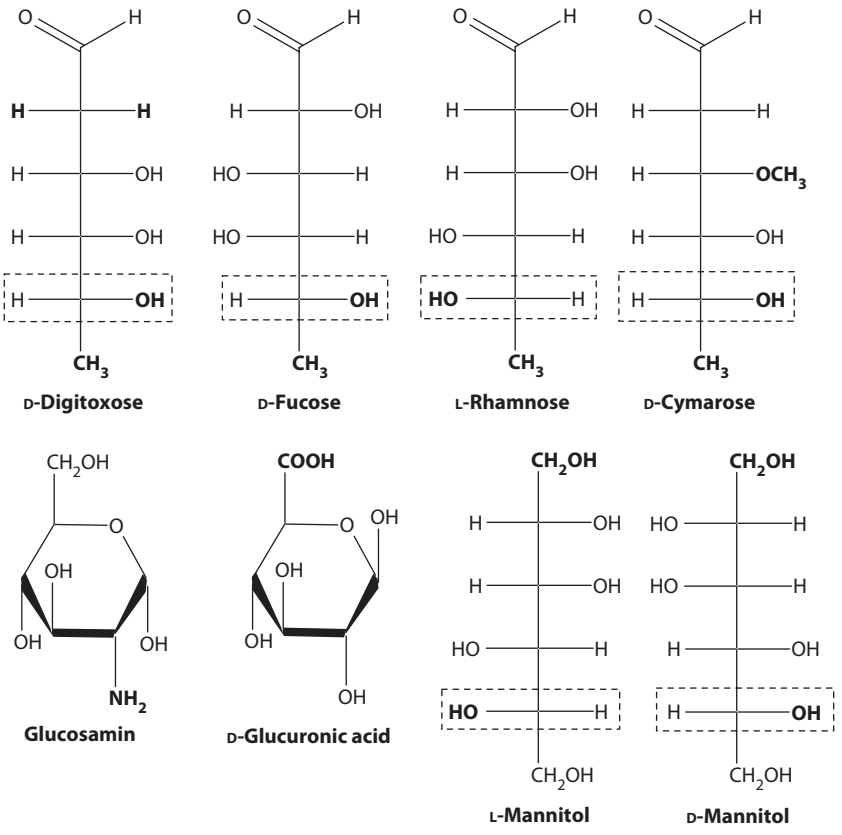


FIGURE 16.6 Modified monosaccharides. The chemical modification is marked by bold capital letters; the position of the hydroxyl group inside the boxes assigns the compound to the L- or D-configuration.

(trehalose family; e.g., saccharose, trehalose), where both sugar units are linked together via their anomeric centers.

16.1.5 Oligosaccharides (Glycans)

Like disaccharides, oligosaccharides are components in which monosaccharide units are joined by glycosidic units. Depending on the number of units they are called disaccharides (two units), trisaccharides (three units), tetrasaccharides (four units), and so on (Figure 16.8). For some of them, trivial names are usually used; for example, the smallest one, α-D-glycopyranosyl-(1→2)-β-D-fructofuranoside, is termed saccharose or sucrose (Figure 16.7). A large number of the known plant (oligo)saccharides are not present in the free state but occur combined with other molecules as plant glycosides. In the majority they form *O*-glycosides (e.g., rutin, primulasaponin); when linked to other atoms, they form *N*-glycosides (e.g., adenosin), *S*-glycosides (e.g., glucosinolates), and *C*-glycosides (e.g., aloin) (Figure 16.9).

Oligosaccharides based on short chains of fructose molecules are found in many vegetables, they are usually abbreviated to FOS (fructose-oligosaccharides). Galacto-oligosaccharides (GOS), which also occur naturally, consist of short chains of galactose molecules. These compounds can only be partially digested by humans but serve as "food" for the intestinal microflora. Functional foods with prebiotic oligofructoses and inulines have become very popular as a dietary ingredient for humans. When oligosaccharides are attached to polypetides, they form glycoproteins, and as such they are often integral membrane proteins and play a role in cell–cell interactions.

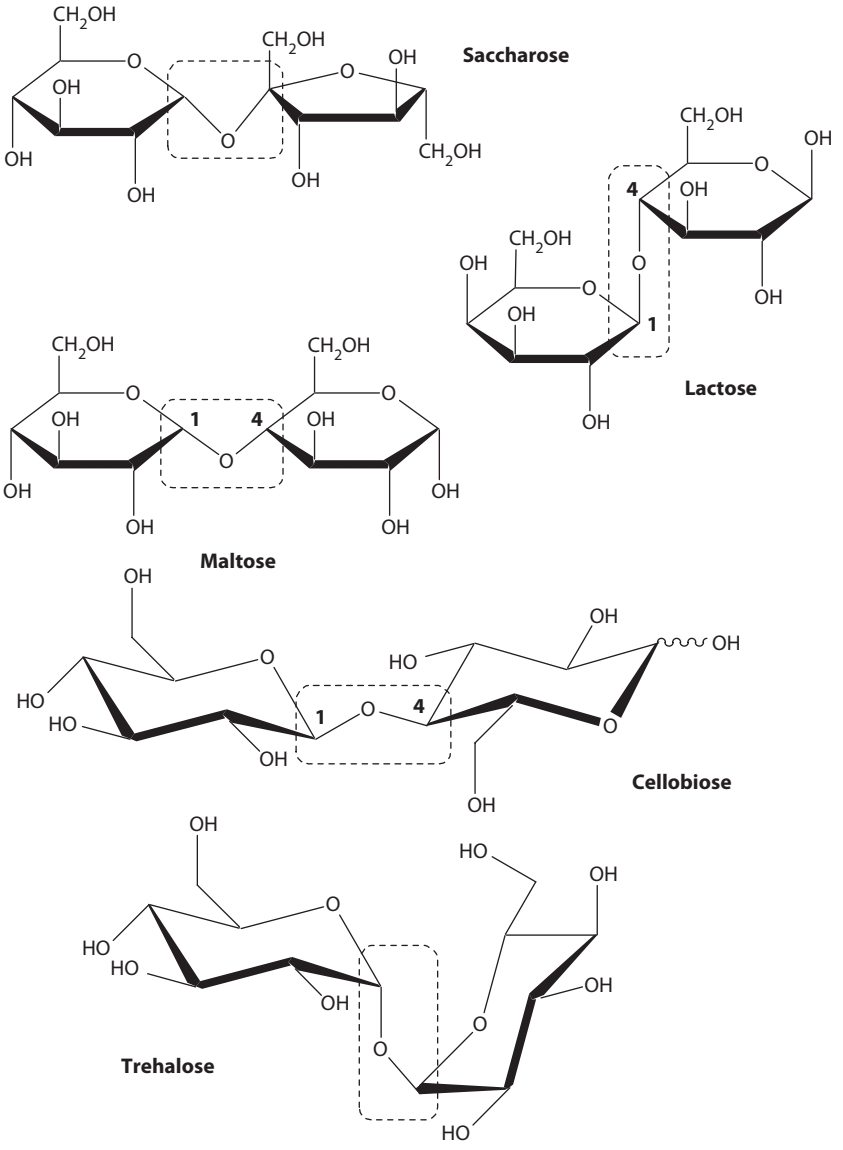


FIGURE 16.7 Examples of disaccharides. Saccharose (2-β-D-fructofuranosyl-1-α-D-glucopyranose), maltose (α-D-glucopyranosyl-(1→4)- α-D-glucoyranose), lactose (D-galactopyranosyl-β-(1→4)-D-glucopyranose), cellobiose (D-glucosyl-β-(1→4)-D-glucopyranose), trehalose (1-α-glucopyranosyl-1-α-glucopyranose).

16.1.6 Polysaccharides

Polysaccharides represent an important class of biological polymers. Their function in living organisms is usually either structure or storage related. Most plant polysaccharides, except for starch and cellulose, are heteropolysaccharides, having more than one type of sugar unit. Starch is used as a storage polysaccharide in plants, being found in the form of both the linear amylose and the branched amylopectin. In animals, the structurally similar but more densely branched glycogen is used instead (Figure 16.10). Fructans are polymers of fructose units. The fructan substance first described in *Inula helenium* (Asteraceae family) was named inulin. Inulin has a much higher degree of polymerization than FOS and is a linear polysaccharide linked via β-(2→1).

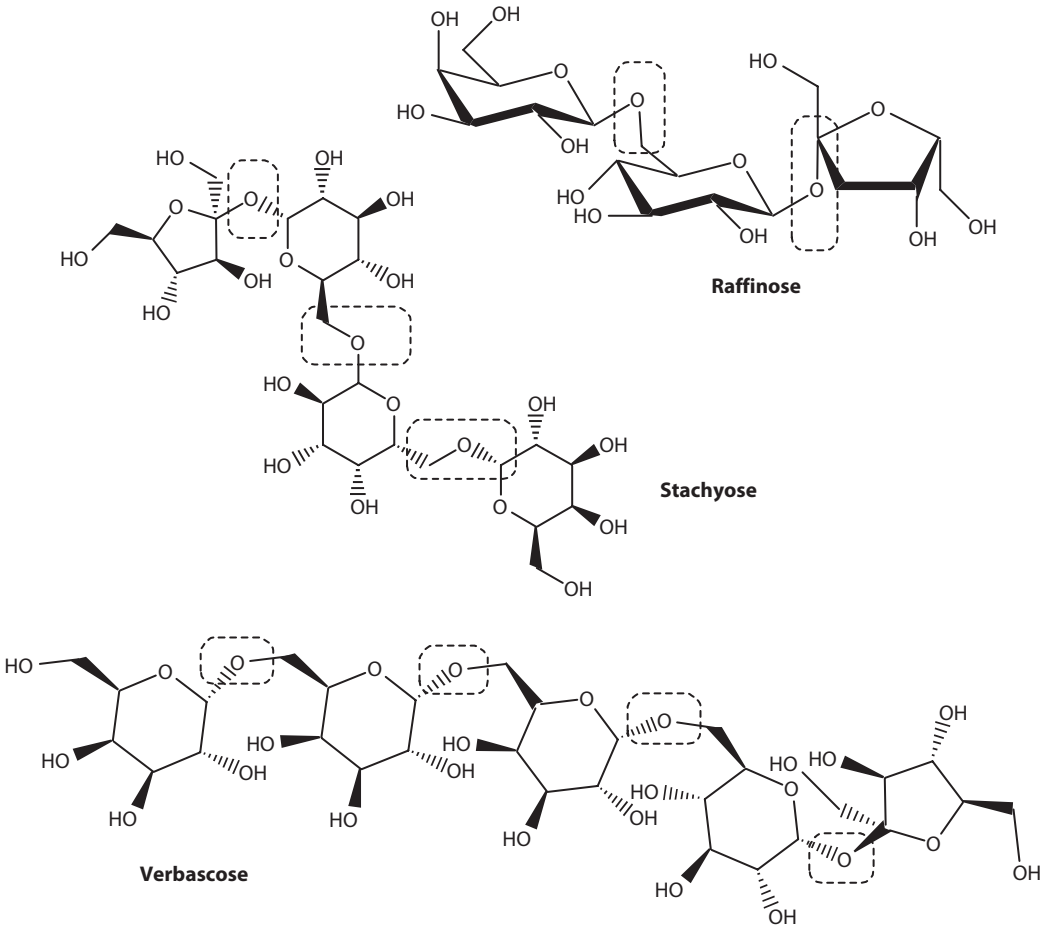


FIGURE16.8 Examplesofoligosaccharides.Raffinose(*O*-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl -(1→2)-*O*-β-D-fructofuranose), stachyose (-D-fructofuranosyl-(2→6)-*O*-α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→6)-α-D-glucopyranose), verbascose (α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranose).

Cellulose and chitin (Figure 16.11) are examples of structural polysaccharides. Cellulose is used in the cell walls of plants and other organisms and is claimed to be the most abundant organic molecule on earth. Chitin has a similar structure to cellulose but has nitrogen (N)-containing side branches, increasing its strength. It is found in arthropod exoskeletons and in the cell walls of some fungi. Compared to cellulose the hemicelluloses contain many different sugars, such as xylose, mannose, galactose, rhamnose, and arabinose. Although a few polysaccharides (e.g., cellulose) are, in fact, simple straight-chain polymers, the majority comprise partly branched structures, such as galactomannans, which consist of a mannose backbone with galactose side chains. Galactomannans are often used in food and the pharmaceutical industry for products to increase viscosity (e.g., guar gum). Glucosaminoglucans are unbranched polymers of alternating uronic acid and glucosamine residues. They play an important role in maintaining extracellular matrix structure. Aminoglycosides belong to a group of natural antibiotics. Peptidoglycans (also known as murein) form the outside membrane of bacterial cell walls. They consist of carbohydrates, such as alternating residues of β–(1→4) linked *N*-acetylglucosamine and *N*-acetylmuramic acid attached to a peptide chain of three to five amino acids (Table 16.1).

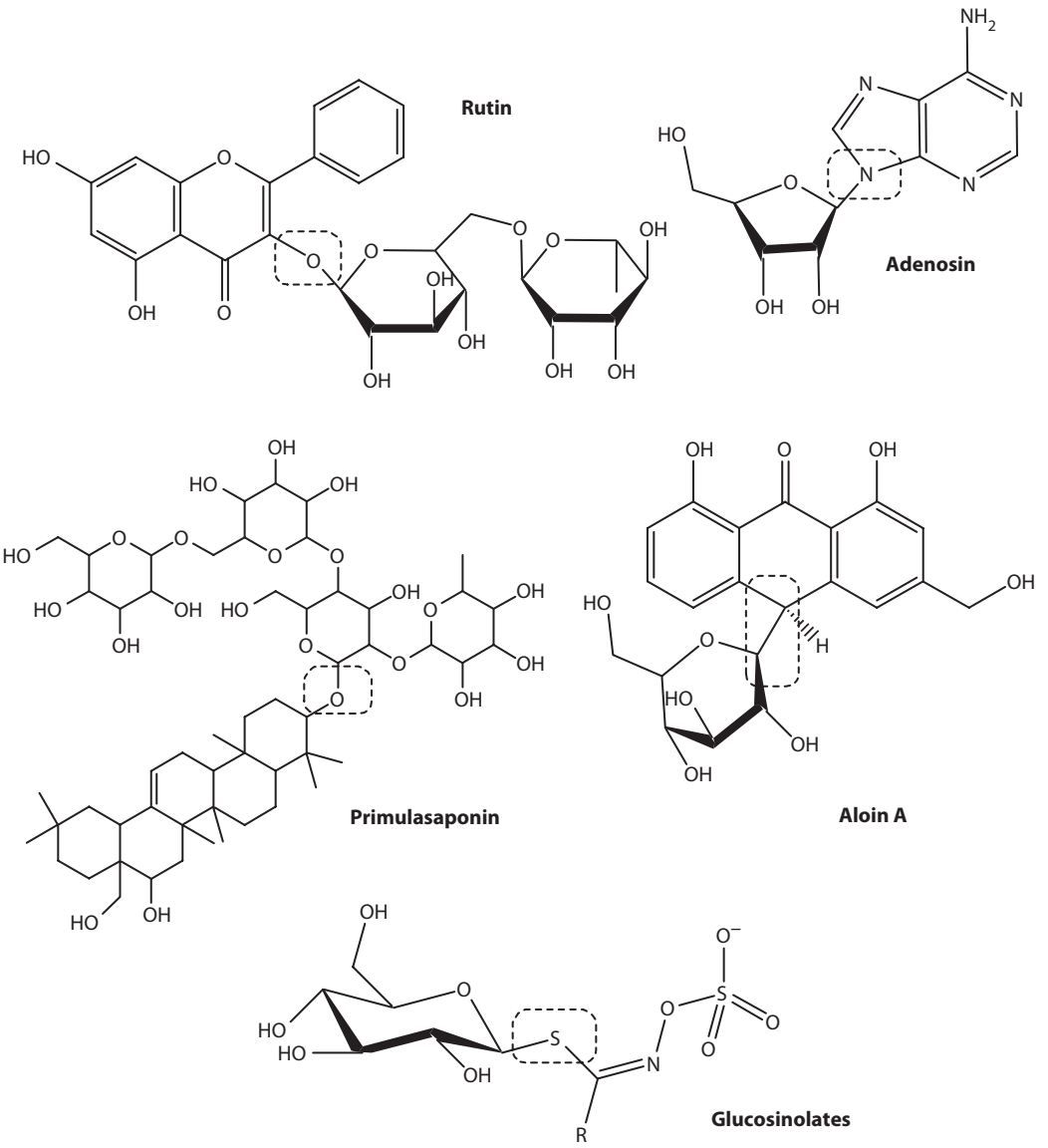
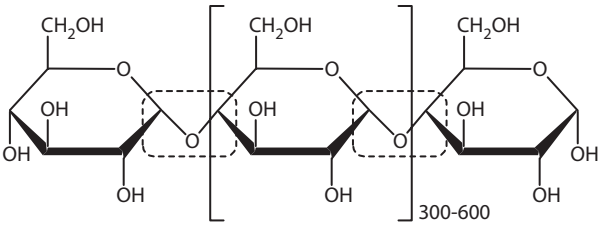


FIGURE 16.9 Plant glycosides (*O*-, *N*-, *C*-, and *S*-glycosides).

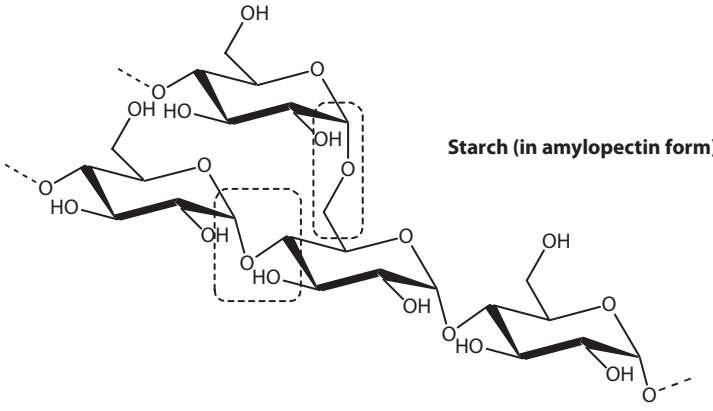
16.2 ANALYSIS OF CARBOHYDRATES

The analysis of mono- and oligosaccharides is of considerable importance to the food and pharmaceutical industries because one important method of ensuring safe food and pharmaceuticals is official monitoring. Because carbohydrates exhibit a significant degree of chemical and physical similarity, they are more difficult to analyze than most other classes of compounds. For polysaccharide analysis, it is, for the most part, only possible to define their structure in terms of repeating smaller units of oligosaccharides. Sugar analysis of the hydrolysate of a polysaccharide or a plant glycoside requires prior removal of the mineral acid used for the hydrolysis. Ion-exchange resins are recommended for removing acid under mild conditions.

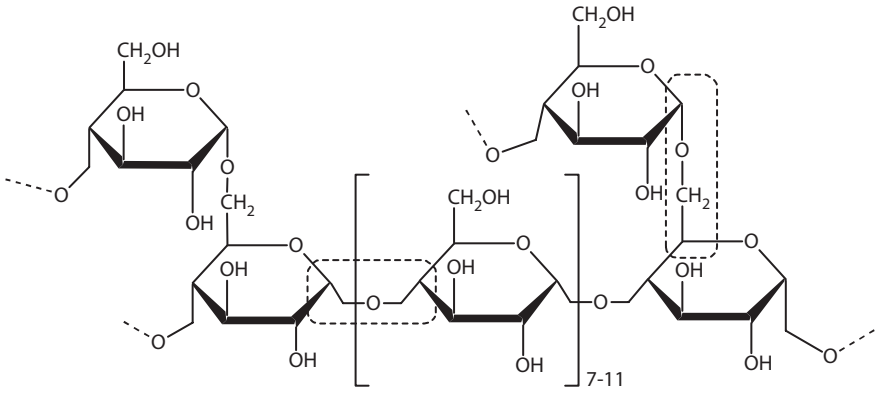
A variety of chromatographic systems including thin-layer chromatography (TLC), gas chromatography (GC), capillary electrophoresis (CE), and high performance liquid chromatography



Starch (in amylose form)



Starch (in amylopectin form)



Glycogen

FIGURE 16.10 Storage polysaccharides. Amylose has α(1→4) linkage of glucose units. Amylopectin, like glycogen, has α(1→4) linkage and α(1→6) linkage where the branching occurs.

(HPLC) can be used to separate and analyze mono- and oligosaccharides. For HPLC various chromatographic modes such as anion or cation exchange, hydrophilic interaction, size exclusion, and reversed phase are routinely used for the resolution of mono- and oligosaccharides. An overview is presented by El Rassi [4].

Because most carbohydrates are hydrophilic and neutral and do not have strong absorbance or autofluorescence to simplify their detection, the aldehyde group at the reducing end or the hydroxyl group can be utilized for *precolumn* or *postcolumn labeling.* Derivatization with appropriate reagents changes these properties and can assist their resolution. For example, it can endow them with charge to facilitate their electrophoretic separation or with hydrophobicity to enable their efficient resolution by reversed-phase HPLC. Furthermore, derivatization of carbohydrates with UV

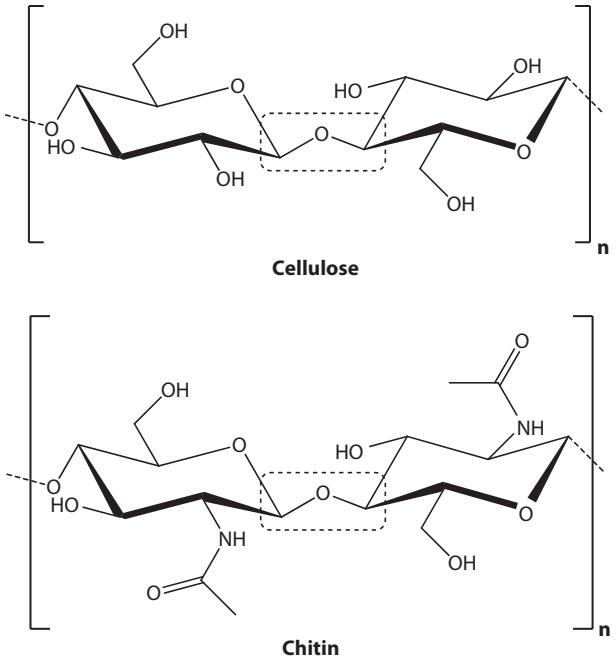


FIGURE 16.11 Structural polysaccharides. Cellulose and chitin are linked via β-(1→4).

TABLE 16.1
Types of Carbohydrates

General		**Aldose, Ketose, Pyranose, Furanose**
Specific		**Open Chain and Ring Formation, Anomer, Mutarotation**
Monosaccharides	Trioses	Ketotriose (dihydroxyacetone)
		Aldotriose (glyceraldehyde)
	Tetroses	Ketotetrose (erythrulose)
		Aldotetroses (erythrose, threose)
	Pentoses	Ketopentose (ribulose, xylulose)
		Aldopentose (ribose, arabinose, xylose)
		Deoxysugar (deoxyribose)
	Hexoses	Ketohexose (fructose, sorbose)
		Aldohexose (glucose, mannose, galactose)
		Deoxysugar (fucose, fuculose, rhamnose)
	Heptose	Sedoheptulose
Multiple Units	Disaccharides	Sucrose (saccharose), lactose, trehalose, maltose, rutinose
	Trisaccharides	Raffinose, gentianose, maltotriose
	Tetrasaccharides	Acarbose, stachyose
	Pentasaccharides	Verbascose
	Other oligosaccharides	Fructooligosaccharide (FOS)
		Galactooligosaccharide (GOS)
		Mannanoligosaccharides (MOS)
	Polysaccharides	Glycogen, starch (amylose, amylopectin) Cellulose, chitin, inulin, dextrin, glucan
	Glycosaminoglycans	Heparin, chondroitin sulfate, hyaluronan, heparan sulfate, keratan sulfate, dermatan sulfate
	Aminoglycosides	Kanamycin, streptomycin, tobramycin, neomycin, gentamicin, amikacin
	Subunit: Macrolides	Erythromycin, roxithromycin

light-absorbing or fluorescent molecules significantly enhances detection sensitivity. Among these methods, reductive amination, which introduces an aromatic amine to the aldehyde group at the reducing end of saccharides, is often preferred (Table 16.2).

The separation of complex mixtures of carbohydrates, ranging from simple monosaccharides to oligosaccarides, is readily achieved using the wide variety of LC columns available. However, most of the HPLC methods reported in the literature on the determination of mono- and oligosaccharides

TABLE 16.2
Derivatization Reagents with Primary Amines for Reducing Carbohydrates

[15]	4-Amino-benzoic acid ethyl ester (4-ABEE)
[43]	2-Amino-3-phenylpyrazine (Yamamoto)
[14]	4-Aminobenzoic acid n-butyl ester (ABBE)
[05]	2-Aminopyridine
[40]	2-Aminbenzamide (2-AB)
[07]	Aminomethylcoumarin
[13]	2-Aminoacridone
[10]	Benzamidin (postcolumn derivatization)
[42]	8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS)

TABLE 16.3
HPLC Conditions and Derivatizations of Mono- and Oligosaccharides

Ref.	Column/Temp.	Mobile Phase	Detection	A: Precolumn, B: Postcolumn	Sample
[6a]	Varian HiPlex Ca, 300 × 7.7 mm, 85°C	Water	ELSD	—	Saccharides
[6a]	Varian HiPlex Ca, 300 × 7.7 mm, 85°C	Water	RI	—	Saccharides
[8]	TSK gel Amide-80, 250 × 4.6 mm, 80°C	Gradient: water –acetonitrile (20%–50%; v/v)	PPD, 400 nm	—	Saccharides of sake
[9]	TSK gel Amide-80, 250 × 4.6 mm	Linear gradient of (A) acetonitrile–H_2O (80:20) to (B) acetonitrile–H_2O (60:40)	UV-fluorescence: 288 nm excitation, 470 nm emission	**B:** Postcolumn fluorescence detection with benzamidin	Oligosaccharides in syrup
[13]	Various ODS phases	5mM NH_4 acetate (pH 6.5)–acetonitrile (30:70)	UV 285 nm, ESI-MS	**A:** Precolumn amination with ABBE or ABEE	Oligosaccharides of maltodextrin
[14]	Spherisorb ODS, 5 µm, 100 × 3.9 mm	17.3 mM SDS–0.01 M phosphate buffer, pH 6.69) and 7.73% (v/v) ethanol (MLC)	UV 307 nm	**A:** Precolumn amination with ABEE, RP_{18}/NH_2 guard columns, 7 µm, 15 mm × 3.2 mm	Sugars of sorghum syrup

Note: ELSD, evaporative light scattering detection; ESI-MS, electrospray ionization–mass spectrometry; MLC, micellar liquid chromatography; PPD, polarized photometric detector; RI, refractive index detector; SDS, sodium dodecyl sulfate.

use normal-phase or reversed-phase columns (Table 16.3) and acetonitrile–water or methanol–water mixtures as mobile phases. Thus, most problems encountered in carbohydrate analysis are those associated with detection rather than the chromatographic separation.

The most commonly used detectors for carbohydrate analysis are refractive index (RI) and UV light detectors. The RI detector, in general, is associated with low sensitivity. Although RI detectors have a wide linear range, they have trouble maintaining stable baselines. In addition, they cannot be used with gradient elution, which may be necessary when analyzing mixtures that contain a wide range of carbohydrates. A UV diode array detector (DAD) allows complete UV spectra to be acquired during the elution of each peak. The evaporative light scattering detector (ELSD) is gaining popularity in today's HPLC. The ELSD offers sensitive detection of any sample that is less volatile than the mobile phase. Unlike UV and RI detectors, the ELSD's response is independent of the sample's optical characteristics. ELSD uses a simple three-step process that produces a signal for any nonvolatile sample, since all particles scatter light: (1) Nebulization performs a dispersion of droplets, (2) evaporation of the mobile phase leads to dry sample particles that are hit with a laser light, and (3) the light is scattered by the particles, generating an electrical signal that—ultimately—is detected. Mass spectrometry (MS) detection using electrospray ionization (ESI) greatly enhances sensitivity and selectivity. Douglas and Vernon [5], for example, permethylated oligosaccharides for sequencing, linkage, and branching studies by MS using an ESI ion trap mass spectrometer.

16.3 APPLICATIONS (MONO- AND OLIGOSACCHARIDES)

16.3.1 Analysis of Mixtures of Sugars and Sugar Alcohols

There are a number of HPLC methods to separate and quantify saccharides. A simple one is mentioned as an example; it involves the use of a calcium ligand-exchange column (e.g., Hi-Plex Ca, Polymer Lab.) with water as a nonpolluting eluent. Nevertheless, an excellent baseline separation of five saccharides could be performed using ELSD (Figure 16.12). Similar good results have been achieved for sugars and sugar alcohol mixtures with an RI detector. The column temperature was, in both cases, set at 85°C (Figure 16.13) [6a].

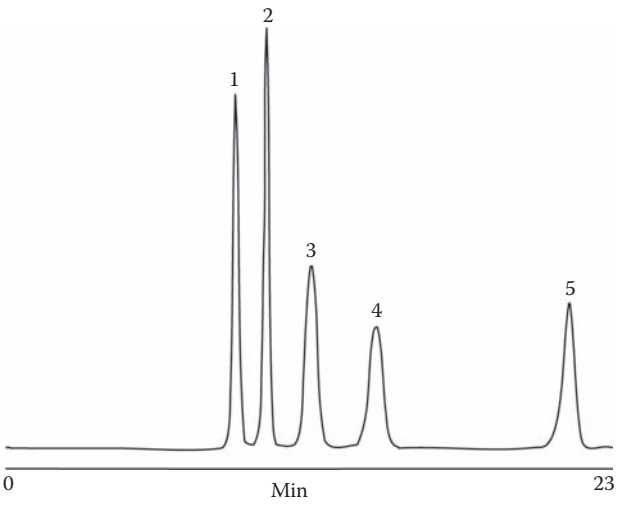


FIGURE 16.12 Varian application note SI-01406. Baseline separation of five saccharides using a Hi-Plex Ca column (9 μm, 300 × 7.7 mm) with the Varian 385-LC ELSD (neb. =30°C, evap. =90°C, gas =1.6 SLM). Key: 1, stachyose; 2, sucrose; 3, glucose; 4, fructose; 5, sorbitol. Sample preparation: saccharides dissolved in water at 1.0 mg/mL; flow rate: 0.6 mL/min., inj. vol. 10 μL, temp: 85°C.

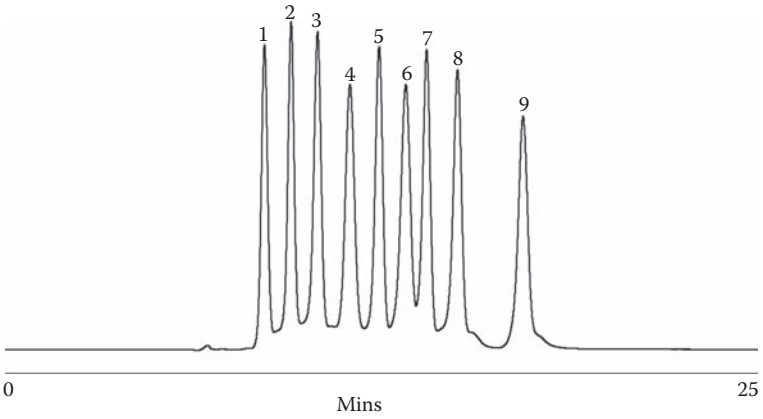


FIGURE 16.13 Varian application note SI-01681. Separation of a mixture of sugars and sugar alcohols using a Hi-Plex Ca (Duo) 8 μm column. The mixture consists of 1, raffinose; 2, sucrose; 3, lactulose; 4, glucose; 5, galactose; 6, fructose; 7, ribitol; 8, mannitol; 9, sorbitol. Sample size: 10 mg/mL; mobile phase: water; flow rate: 0.4 mL/min; injection volume: 10 μL; temp.: 85°C. Detection: Varian 356 – LC RI Detector.

16.3.2 Analysis of Sake

Recently, several oligosaccharides were ascertained to have biological functions, such as improvement of intestinal flora by stimulation of *Bifidobacteria* growth or enhancing immune function in humans. Sake is an alcoholic beverage brewed from rice. The koji mold (*Aspergillus oryzae*) produces enzymes that convert rice starch to sugars such as glucose and maltose, and sake yeast (*Saccharomyces cerevisiae*) metabolizes the sugar to ethanol; in addition, sake contains unfermentable oligosaccharides such as isomaltose and panose. Sake may also be useful for healthy nutrition; for example, the number of *Bifidobacteria* in human feces increased after administering isomaltooligosaccharides to (healthy) humans. For quality purposes it is important to discriminate fermentable saccharides from unfermentable saccharides in sake.

In the early 1990s several HPLC methods were reported for the simultaneous determination of mono- and oligosaccharides using a normal-phase column and an RI detector [6b,7]. Unfortunately they resulted in bad or incomplete separation of either maltose–isomaltose or maltotriose–isomaltotriose–panose. That is the reason why an HPLC method has been developed on a carbamoyl (NH_2) bonded silica column using a linear gradient elution of water in acetonitrile [8]. Saccharides were determined by a special polarized photometric detector (PPD). The PPD enables the measurement of the optical rotation of chiral compounds as a change in absorbance. The proposed method did not require any sample cleanup treatment when saccharide compositions in various kinds of sake, such as "synthetic" ones and samples produced by fermentation as described previously, were compared. Seven saccharides, ranging from mono- to trisaccharides, and ethyl α-D-glucoside were determined. The "synthetic" sakes appeared to be composed of glucose and maltose, whereas the "original" sake contained glucose, various unfermentable saccharides, and a high concentration of ethyl α-D-glucoside and, obviously, had a better taste. Another solution to the analysis of an isomaltose-oligosaccharide syrup is an HPLC technique using postcolumn fluorimetric detection [9]. An acetonitrile–water gradient separated the reducing mono- and oligosaccharides simultanously on an NH_2-bonded column.

16.3.3 Analysis of Maltodextrin

Maltodextrin is a nonsweet, nutritive complex carbohydrate consisting of dextrose (glucose), maltose, maltotriose, and higher polysaccharides. It is prepared by the partial hydrolysis of food-grade

starch with suitable acids and/or enzymes. The degree of hydrolyzation is expressed in dextrose equivalents (DE = the degree of conversion of the starch into the sugar dextrose). According to the monographs of the U.S. Pharmacopeia and National Formulary (USP/NF) [10] and the European Pharmacopoeia (PhEur) [11], maltodextrin consists of less than 20 DE. Maltodextrin is recommended for those (patients) whose metabolic rate and appetite is the limiting factor in their ability to gain weight. Furthermore, maltodextrin is used in a wide variety of products for customers stretching from bodybuilders, to animal trainers, to food, and pharmaceutical manufacturers. Quality assurance, according to the monographs of USP/NF and PhEur as well, is very time consuming, but no further particulars are offered.

HPLC analysis of oligosaccharides using online detection with a mass spectrometer represents a straightforward approach to obtain valuable information on the composition of a glycan pool. Conventional reversed-phase HPLC and subsequent analysis of fractions by electrospray ionization (ESI)-MS and MALDI-TOF-MS represent a powerful approach to the analysis of derivatized oligosaccharides since an inherent disadvantage of HPLC with UV/VIS detection is the very limited structural information it offers [12].

The separation of oligosaccharides by reversed-phase HPLC depends strongly on the nature of the stationary phase. Four different octadecyl silane (ODS) phases were investigated for their ability to separate homologous maltodextrins (malto-oligosaccharides), which had been derivatized with aminobenzoic acid ethyl ester (ABEE) or aminobenzoic acid *n*-butyl ester (ABBE) [13]. UV/VIS detection was used (285 nm for the aminobenzoic esters) in combination with on-line ESI triple-quadrupole MS and off-line matrix assisted layer desorption/ionization- time of flight (MALDI-TOF)-MS. The highest efficiency was found using ODS-3 CP as stationary phase. In the same range of linear flow velocity, ODS-1 PE showed the poorest performance. In all cases, the elution order of the modified oligosaccharides was found to be inversely related to the degree of polymerization; that is, the larger carbohydrates eluted earlier than smaller ones containing less glucose units. Excess derivatization reagent eluted after glucose, the last sugar to be eluted, and thus did not interfere with the separation of the oligosaccharides. Malto-oligosaccharides with a degree of polymerization of up to 25 could be resolved to baseline. These results were compared to the outcome using an early capillary HPLC (named nano-HPLC) system with on-line coupling to ESI-MS. Though this new system demanded some modifications of the commercial interface—caused by the low flow rates in capillary HPLC—it represented a powerful tool for the analysis of ABBE- or ABEE-derivatized oligosaccharides.

16.3.4 Analysis of Sorghum Syrup

Sorghum syrup is a natural sweetener made by processing juice squeezed from the seed or stalks of certain types of sorghum (*Sorghum bicolor*), called sweet sorghum or sorgo. Sweet sorghum is grown for syrup or feed, whereas most other sorghums, commonly referred to as milos or kafirs, are grown for grain. Sweet sorghums resemble grain sorghum at maturity except they are approximately three times taller, reaching a height of 4 m or more.

Momenbeik and Khorasani [14] reported the separation and determination of sugars by micellar liquid chromatography (MLC). Micelles are composed of surfactants, and the addition of micelles to the mobile phase introduces a third phase into which the solutes may partition. The conditions, including sodium dodecyl sulfate (SDS) concentration, pH, and amount of ethanol in the mobile phase, had to be optimized. The optimized method was applied to the analysis of sugars in a sample of sorghum syrup, and results were compared with those obtained by HPLC on an NH_2 column with RI detection and 75% acetonitrile in water as the mobile phase. The sugars were derivatized with 4-ABEE. There was good agreement between the results obtained by use of the proposed method (MLC) and those obtained by conventional HPLC (Table 16.3).

16.4 POLYSACCHARIDES AS ADDITIVES AND EXCIPIENTS IN FOODS AND PHARMACEUTICALS

The detailed analysis of the polysaccharide hydrocolloids added to foods as thickeners, gelling agents, or stabilizers is a great challenge for the analyst because, in general, hydrocolloids are used at low levels (0.01–1%) in a food matrix that, again, often contains closely related polysaccharides (starch or pectins) in much greater quantities than the added hydrocolloids.

16.4.1 Polysaccharides Derived from Algae

During the last several decades there has been an increasing interest in the commercial production of food-grade microalgae for human consumption, and studies have reported enhanced immune function in both animals and humans. Among the various microalgae that have been explored for their commercial potential as food supplements, *Spirulina* species, *Chlorella* species, and *Aphanizomenon flos-aquae* (AFA algae) are three major types that are in widespread use. The active components for all these effects have been established to be high-molecular-weight polysaccharides. From *Spirulina* and *Chlorella* but not from *Aphanizomenon flos-aquae* species, a number of polysaccharides have been characterized for their antitumor, antiviral, and immunostimulating activity. (*Aphanizomenon flos-aquae*-products are often contaminated with microcystin, a cyanobacterial toxin.)

Based on their glycosidic compositions, glycosidic linkages, and molecular weights, microalgae polysaccharides are determined, and the combination of the individual polysaccharides is for the most part protected by patent. One paper [15] is cited as an example for the analysis of high-molecular-weight polysaccharides. In this study the ethanol extracts of the freeze-dried microalgae were analyzed using size-exclusion chromatography (SEC) combined with RI detection using water as the mobile phase. Estimation of the molecular weight for each peak was achieved by comparison with retention times for dextran standards. In an earlier publication [16] a combination of reversed-phase HPLC with DAD and precolumn derivatization was presented to quantify the agar-type polysaccharides. This method enabled a separation of anomeric forms of α- and β-galactose.

16.4.2 Analysis of Sulfated Polysaccharides of Marine Algae

Sulfated polysaccharides are polysaccharides whose monomers are esterized to sulfuric acid residues and are moreover partially methylated. They have been detected in nearly all marine algae. They occur partially in the cell wall itself and partially in the intercellular substance. Sulfated galactanes are typical for many red algae. Depending on their origin they are called, for example, agarose, carrageenan, porphyran, and furcelleran. L- and D-galactose, which are linked by β-(1→3) or α-(1→4) glycosidic bonds, form the basic pattern of agarose and porphyran; in the latter L- and D-galactosyl residues alternate. Carrageenan and furcelleran contain exclusively D-compounds.

Polysaccharide hydrocolloids are polydisperse molecules. As a result, there is no general procedure for their analysis in mixed food systems, but various techniques that may be roughly divided into two main categories: (a) techniques that necessitate polysaccharide depolymerization procedures such as reduction of acidic function and peracetylation or permethylation with subsequent chromatographic analysis of individual sugars released; or (b) techniques without depolymerization prior to qualitative or quantitative analysis.

In the second category, techniques that were applied with some success were based either on electrophoresis or on immunological techniques. Enzyme linked immuno sorbent assay (ELISA) are generally highly sensitive but require also highly specific antisera in order to avoid cross-reactions.

The enzyme-linked lectin assay (ELLA) technique specifically detected the galactomannans in commercial food products but suffered from inhibitions by sugars such as galactose and lactose to varying extents depending on the sugar nature and concentration.

Alternatively, the hydrolysis and methanolysis methods quantify monosaccharides from hydrocolloids, and the relative proportion of the constitutive sugars indicates the presence of individual or mixed polysaccharides. However, the choice of conditions for aqueous hydrolysis, in particular for anionic gums such as alginates and carrageenans, is critical due to the various stabilities of the released monosaccharides in the acidic medium. Methanolysis presents the advantages of better stability of released methylglycosides and of a simultaneous analysis of acidic and neutral sugars by capillary GC and HPLC.

Carrageenans are water-soluble sulfated galactans built on the repetition of 1→3-linked β-D-galactopyranose alternating with 1→4-linked α-galactopyranose. The latter sugar often occurs as 3,6-anhydro-D-galactose. The sulfation pattern of carrabiose is the basis of carrageenan classification, and mainly three types of carrageenans are used industrially, that is, lambda, kappa, and iota. Carrageenans or carrageenins are extracted from red seaweeds (algae). Gelatinous extracts of the red algae *Chondrus crispus* (L.) STACKH. and *Girgatina stellata* (STACKH.) BATT. have been used as food additives for hundreds of years, though analysis of carrageenan safety as an additive continues.

The method presented by Quemener *et al*. [17] proved to be reliable for specific determination of carrageenans, used alone or with other hydrocolloids, namely, pectins, alginate, xanthan, or galactomannans in complex food products such as yogurt. Methanolysis performed in mild conditions and coupled to reversed-phase HPLC was optimized using water as the mobile phase and RI detection. This specificity relies on the determination of the characteristic 3,6 anhydrogalactose present only in gelling κ (kappa) and ι (iota) carrageenans and not in λ (lambda) carrageenan.

16.4.3 Analysis of Bacterial Polysaccharides

Most microorganisms produce exopolysaccharides, which can be excreted or covalently bound to the cell surface as capsular polysaccharides. In the field of vaccine research these saccharides play a pivotal role. Carnago *et al*. [18] presented an HPLC method to monitor the capsular polysaccharide type b of *Haemophilus influenzae*. The separation was performed on a Sepharose column with water as eluent and RI and UV detection.

Xanthan gum (E415) is a high-molecular-weight polysaccharide gum produced by a pure-culture fermentation of a carbohydrate with the gram-negative bacteria *Xanthomonas campestris*. This natural polysaccharide is widely used in the food industry and to a lesser extent in the pharmaceutical industry. Xanthan gum is monographed in the USP28/NF [10] and in the PhEur [11]. It is soluble in hot and cold water, as well as being stable under acidic and alkaline conditions (pH 5–13).

The primary structure of xanthan gum contains D-glucose and D-mannose as the dominant hexose units, along with D-glucuronic acid. The trisaccharide side chain consists of two D-mannose residues and one D-glucuronic acid residue occurring as mixed K^+, Na^+, and Ca^{++} salts. Through the association of xanthan molecules, it is thought that a quaternary structure arises through the charged trisaccharide side chains. Xanthan gum is a water-soluble polymer. Neither USP nor PhEur provides an HPLC method for determination of identity or purity.

The sugar composition of xanthan is difficult to obtain as the backbone, which like cellulose consists of β-(1→4) linked D-glucose units, is highly resistant to hydrolysis. Due to these problems, the official description of xanthan, for example, by the Joint Expert Committee for Food Additives (JECFA) [19], does not refer to its chemical composition but only to its ability to gellify in the presence of locust bean gum. In the official description, there is no reference to acetyl groups, only to pyruvic acid (Table 16.4).

TABLE 16.4
HPLC and Separation Conditions of Polysaccharides

Ref.	Column/Temp.	Mobile Phase	Detection	Sample Pretreatment	Sample
[15]	Shodex Ohpak KB-805, 300 × 8 mm, 30°C	Water	RI	SEC	Dextran units of microalgae
[17]	Merck Superspher RP_{18}, 15°C	Water	RI	Methanolysis	Carrageenans
[18]	6 HR 10/30 Sepharose	Water	RI, UV 206 nm, UV spectra	—	Bacterial polysaccharides

Note: RI, refractive index detection; SEC, size-exclusion chromatography.

16.5 ANALYSIS OF ANTIBIOTICS

Antibiotics are substances produced by some microorganisms that kill other microorganisms or inhibit their growth, for example, by inhibiting the synthesis of bacterial cell walls. Bacteria are a large group of unicellular microorganisms. Since they lack the nucleus and organelles of the more complex cells called "eukaryotes," bacteria are considered to be "prokaryotes." Surrrounding the bacterial cell membrane is the bacterial cell wall, which characteristically contains peptidoglycan (or murein in older sources). Peptidoglycan is unique to procaryotic cells and is composed of cross-linked chains of identical peptidoglycan monomers. The sugar component consists of alternating residues of β-(1→4) linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues. Attached to the *N*-acetylmuramic acid is a peptide chain of three to five amino acids. The peptide chain can be cross-linked to the peptide chain of another strand forming a 3D meshlike layer. The peptidoglycan layer (Figure 16.14) is substantially thicker in gram-positive bacteria than in gram-negative bacteria, with the attachment of the surface layer. The names *gram-positive* and *gram-negative* originate from the reaction of cells to the Gram stain, a test proved for the classification of bacterial species. Gram-negative bacteria have a relatively thin cell wall consisting of a few layers of peptidoglycan that are surrounded by an outer membrane containing lipopolysaccharides and lipoproteins. The cell wall is essential to the survival of many bacteria, and some antibiotics (e.g., penicillin) are able to kill bacteria by inhibiting a step in the synthesis of peptidoglycan. Another group of antibiotics may affect protein synthesis, which takes place in the ribosomes of the bacteria. Since ribosomes are different in eucaryotes and procaryotes, this difference provides the basis for the selective antimicrobial action of some antibiotics. The bacterial ribosome consists of a 30S subunit and a 50S subunit, whereas in mammalian ribosomes the subunits are 60S und 40S. The other elements involved in peptide synthesis are messenger RNA, which forms the template for protein synthesis, and transfer RNA, which brings the individual amino acid to the ribosome.

Antibiotics generally have no chromophore and need a thorough sample cleanup [20] or have to be derivatized in order to obtain UV-absorbing or fluorescent compounds. These derivatization steps conducted in the precolumn [21,22] or postcolumn mode [23,24] allow a satisfactory sensitivity and selectivity to be reached for analysis of residues in complex biological matrices (Table 16.5).

16.5.1 Analysis of Roxithromycin—Enhancement of Sensitivity by Sample Cleanup

Roxithromycin is a semisynthetic macrolide antibiotic (Figure 16.15) derived from erythromycin. Roxithromycin binds to the subunit 50S of the bacterial ribosome and thus inhibits the translocation of peptides. Roxithromycin has a similar antimicrobial spectrum to erythromycin but is more

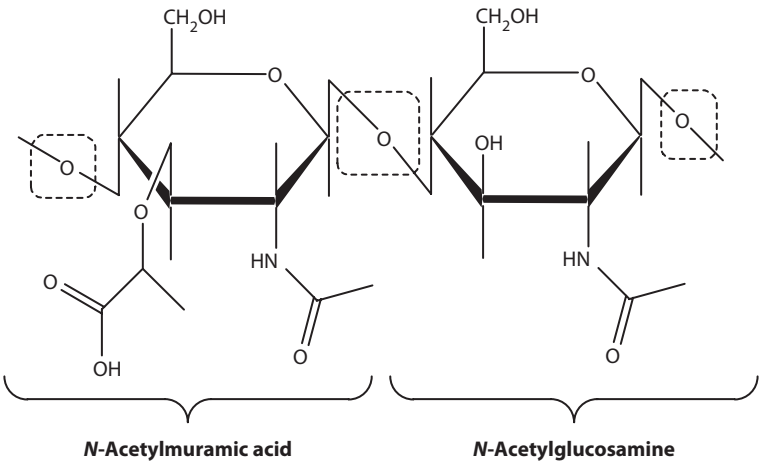


FIGURE 16.14 Peptidoglycan layer in bacteria. There is β-(1→4) linkage between the *N*-acetylmuramic acid and *N*-acetylglucosamine units.

TABLE 16.5
HPLC Conditions of Aminoglycoside Antibiotics and Macrolides

Ref.	Column/Temp.	Mobile Phase	Detection	A: Derivatization, B: Guard Column/ Cleanup	Sample
[20]	Nucleosil 100 C18, 3 μm, 150 × 4.6 mm, 60°C	Methanol–15 mM KH_2PO_4 buffer (pH 6.0) (70:30, v/v)	UV 220 nm	B: Nucleosil 120 C18, 5 μm, 10 × 4 mm	Roxithromycin in human plasma
[21]	Purospher RP18e, 3 μm, 55 × 4 mm	Methanol–water (67:33, v/v)	UV 230 nm	A: Precolumn derivatization with NITC	Kanamycin A
[22]	MicroPak SP C_8, 150 × 4 mm	0.02M phosphate buffer (pH 6.5)–acetonitrile (52:48, v/v)	Fluorescence: Ex. 340 nm, Em. 450 nm	A: Precolumn derivatization with OPA	Tobramycin
[23]	Hypersil BDS C18e, 3 μm, 100 × 4 mm	10 mM AHS, 0.4 mM NQS in 20% AN–AN (97:3)	Fluorescence: Ex. 260 nm, Em. 435 nm	A: Postcolumn derivatization with 0.2M NaOH, 55°C B: Hypersil ODS, 5 μm, 4 × 4 mm	Streptomycin

Note: AN, acetonitrile; AHS, sodium 1-heptane sulphonate; NITC, 1-naphthyl isothiocyanate; NQS, 1,2-naphthoquinone-4-sulphonic acid sodium salt; OPA, orthophthalaldehyde.

effective against certain gram-negative bacteria, particularly *Legionella pneumophila*. It can treat respiratory tract, urinary, and soft tissue infections.

Several chromatographic methods have been reported for the determination of roxithromycin in biological fluids, such as chromatography on RP columns and electrochemical detection. The unavoidable sample pretreatment consisted of liquid–liquid extraction or was based on column switching [26]. In comparison with other macrolides roxithromycin has somewhat higher absorbance at lower wavelengths. Therefore a method with spectrophotometric detection should be feasible, because a limit of quantitation 0.5 μg/mL is sufficient for pharmacokinetic studies. The

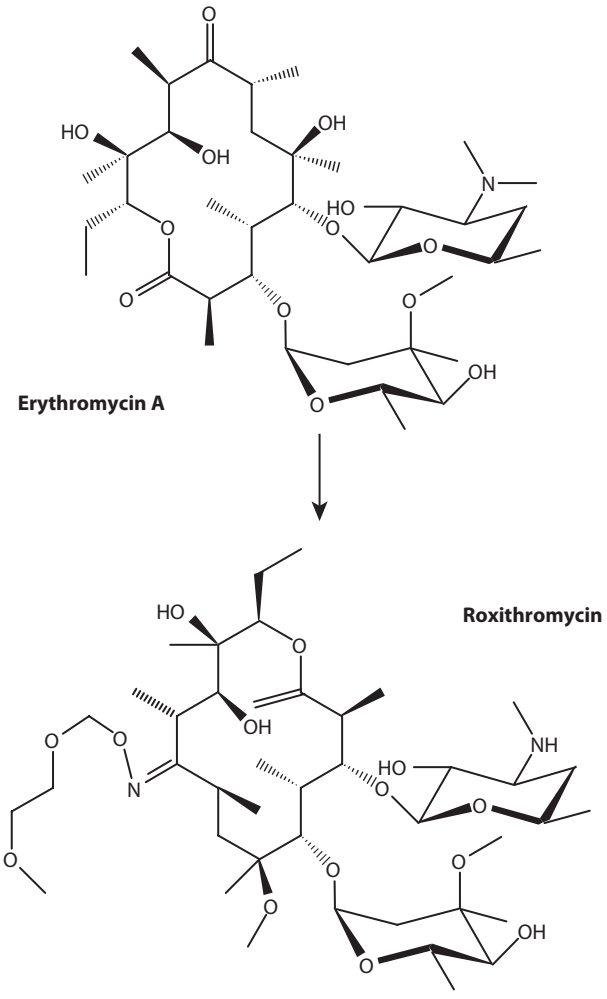


FIGURE 16.15 Roxithromycin.

proposed method [20] was applied to the determination of roxithromycin in plasma samples using a RP18 column and UV detection at 220 nm. The mobile phase consisted of methanol–15 mM potassium dihydrogenphosphate buffer (70:30, v/v); the pH of the buffer was adjusted to 6.0 with potassium hydroxide. The flow rate of the mobile phase was 1.2 mL min^{-1} at 60°C.

16.5.2 Analysis of Streptomycin—Enhancement of Sensitivity by Postcolumn Derivatization

Streptomycin (Figure 16.16) is an aminoglycoside antibiotic drug, derived from the actinobacterium *Streptomyces griseus*. It was the first antibiotic remedy for tuberculosis. It kills sensitive bacteria by inhibiting protein synthesis; more specifically, it binds to the 16S rRNA (a component of the bacterial ribosomal 30S subunit). This prevents initiation of protein synthesis and leads to death of microbial cells. Humans have structurally different ribosomes from bacteria, thereby allowing the selectivity of this antibiotic for bacteria. Streptomycin cannot be given orally but must be administered by regular intramuscular injection. An adverse effect of this medicine is ototoxicity, which can cause temporary hearing loss.

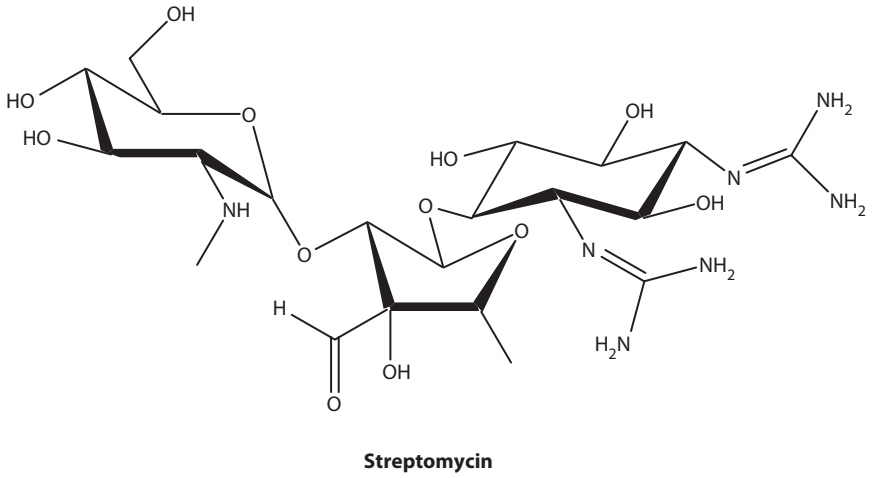


FIGURE 16.16 Streptomycin.

Streptomycin is used as a veterinary drug, especially in beekeeping, for the treatment of bacterial diseases, such as American or European foulbrood. Therefore, streptomycin residues can be found in meat, liver, kidneys, milk, and, above all, honey. Though the streptomycin concentrations found in food have no direct toxic effect, numerous allergic hypersensitivity cases were discovered during recent years. Streptomycin microbial resistance is a common finding, and it has been shown that *Escherichia coli*, *Salmonella*, and *Shigella* may carry resistance. For all these reasons, the control of streptomycin residues in food is necessary.

Consequently, a specific, sensitive, and reliable analytical method is needed. Microbiological methods and the Charm test [26] are not sensitive or specific enough. Immunological assays are very sensitive and can be used as screening tests, but results obtained with these assays must always be confirmed by a more selective method such as chromatography. Streptomycin has no chromophore and is generally derivatized in order to obtain UV light-absorbing or fluorescent compounds. This derivatization step allows a satisfactory sensitivity and selectivity to be reached for the analysis of residues in complex biological matrices.

A sensitive procedure was presented for the analysis of streptomycin in food of animal origin, like meat, milk, and honey [23]. The method is based on a separation by ion-pair liquid chromatography with β-naphthoquinone-4-sulfonate (NQS), postcolumn derivatization, and fluorescence detection. The cleanup of the extract was done by solid-phase extraction (SPE), first with a cation-exchange cartridge and second with an octadecyl cartridge. The selectivity was very good, and few interfering peaks were observed for various food matrices. The streptomycin recovery of the total procedure was greater than 80%. A special focus was given to streptomycin residues in honeys, and a survey of 64 commercial honeys was presented.

After the two-phase sample cleanup the chromatographic separation was performed in an isocratic mode with a RP18 column. The reaction temperature was 55°C inside the reaction coil. The fluorometric detection was done at 260 nm excitation and 435 nm emission wavelength.

16.5.3 Analysis of Kanamycin and Tobramycin—Enhancement of Sensitivity by Precolumn Derivatization

Kanamycin (Figure 16.17), an aminoglycoside antibiotic, is produced naturally during fermentation of *Streptomyces kanamyceticus*. It is commonly administered for the treatment of serious gram-negative and gram-positive bacterial infections. Its activity against *Mycobacterium tuberculosis*

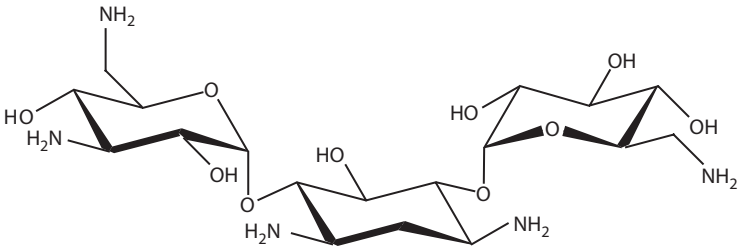


FIGURE 16.17 Kanamycin A.

has made it a useful tool as a second-line drug in the treatment of tuberculosis in combination with other effective drugs.

Kanamycin interferes with protein synthesis in the bacterial cell. The bactericidal activity of kanamycin is attributable to irreversible binding to 30S and 50S ribosomal subunits, resulting in defective protein synthesis. Kanamycin contains kanamycin A as the main active component, and only small amounts of structurally related components such as kanamycin B and C. The structure of kanamycin A indicates that kanamycin A has four primary amino and seven secondary OH groups. Like the other aminoglycosides, kanamycin A has a comparatively narrow safety margin. The therapeutic plasma concentration of kanamycin A is in the range of 10–30 μg/L, and aminoglycoside antibiotics may exhibit severe ototoxicity and nephrotoxicity in long-term therapy. Therefore, monitoring of aminoglycoside antibiotic levels in plasma is required for therapeutic and toxic control.

HPLC techniques are the most widely used methods for the analysis of kanamycin A, and HPLC with electrochemical detection has been applied to assay kanamycin A in bulk and pharmaceutical preparations according to US Pharmacopeia 28 [10]. These official methods are not suitable for measuring low levels of antibiotics in plasma. Kanamycin A lacks suitable chromophores capable of generating a reliable signal in the UV region. However, in order to increase the UV absorption of the kanamycin A molecule, chemical precolumn derivatization of the primary amino groups with 1-naphthyl isothiocyanate (NITC) to form naphthyl isothiourea derivatives was carried out. The reaction had to be performed under mild conditions to avoid hydrolysis and/or degradation of the molecule, and methylamine was used to eliminate excess NITC after derivatization. The application of the method for monitoring kanamycin A in humans upon intramuscular injection demonstrated the usefulness of the assay for clinical studies.

Tobramycin, a further aminoglycoside antibiotic, is a good example of a polar pharmaceutical compound with low selectivity and poor recovery. To improve its cleanup from a biological matrix, a method has been developed using pre-SPE derivatization with orthophthalaldehyde (OPA). This method has the advantage of enhanced detection sensitivity by means of fluorescence detection of the derivative formed [22]. Due to multiple primary amino sites on the compound, multiple derivatives may be expected. In fact, two derivatives are obtained. The ratio of derivative 2 to derivative 1 increases with reaction time and with increases in acetonitrile in the sample solvent. The optimum reaction time is 30 minutes, and the optimum solvent composition is 60% acetonitrile. There is no interference from the blank. Using fluorescence, the detection limit is about 400 times better than when using UV absorbance (Table 16.5).

16.6 ANALYSIS OF GLUCOSAMINOGLYCANS

Oligosaccharides derived from glucosaminoglucans and proteoglycans are a diverse set of molecules with significant size, charge, composition, and linkage heterogeneity. Thus, as reviewed [27,28], reversed-phase chromatography has been applied using aqueous organic solvents to separate neutral oligosaccharides, or using ion-pair reagents for sulphated oligosaccharides. Normal-phase amine-bonded chromatography has been applied to neutral oligosaccharides in aqueous organic solvents

separated by various buffer systems. High-pH anion-exchange chromatography has its primary applications for N-linked chains but can also be used for neutral reducing oligosaccharides and neutral or sulphated alditols [29–32], chondroitin disaccharides [33], and oligosaccharides of HA [34]. Multiple sulphated oligosaccharides have been mainly chromatographed on strong anion-exchange columns [35,36] or reversed-phase columns [37,38] with elution in nonvolatile buffer systems. The further analysis of oligosaccharides is often dependent on salt-free samples, and thus the use of volatile buffers for purification is an important consideration.

Davies and Hounsell [39] explored porous graphitized carbon (PGC) for HPLC of mono- and disaccharides released from proteoglycans, and they optimized the detection of fluorescently labeled oligosaccharide derivatives. Sulphated oligosaccharides showed good retention and separation behavior on PGC-HPLC, and compared to anion-exchange or reversed-phase ion-pair chromatography, the chromatography was carried out in the absence of salt. Their research has confirmed that fluorescence derivatives offer a highly sensitive alternative that relies on reductive amination methods.

Chondroitin sulfate is usually found attached to proteins as part of a proteoglycan. A chondroitin chain can have over 100 individual sugars, each of which can be sulfated in variable positions and quantities. Understanding the functions of such diversity in chondroitin sulfate and related glycosaminoglycans is a major goal of glycobiology. Chondroitin sulfate is an important structural component of cartilage and provides much of its resistance to compression. Along with glucosamine, chondroitin sulfate has become a widely used dietary supplement for treatment of osteoarthritis.

Chai et al. [40] made an approach to derive an array of oligosaccharide fragments of defined and unmodified structure that conform to regions of the original polysaccharide chains. This approach depended on the high specifity of lyase digestion combined with subsequent cleavage of the terminal unsaturated uronic acid by an oxymercuration reaction. The depolymerized material was fractionated by gel filtration, and the formed tri- to octasaccharide fractions were further chromatographed by HPLC on a column of amino-propyl bonded silica and monitered by UV absorbance at 206 nm. Depending on the size of the oligosaccharides, different linear gradients of NaH_2PO_4 were performed. Finally, the oligosaccharides were characterized by MS and ^{1}H-NMR.

Hyaluronic acid (HA) is an ubiquitous glycosaminoglycan that plays an important role in maintaining extracellular matrix structure and in regulating intercellular activities like cell–cell attachment and aggregation through its engagement of membrane-bound ligands (Figure 16.18).

In recent years, there has been a considerable increase in research on HA molecules as more and more of its functions in physiology and pathology have become known. A key finding in this field is that HA oligosaccharides or small polymer "fragments" can stimulate angiogenesis, a process that

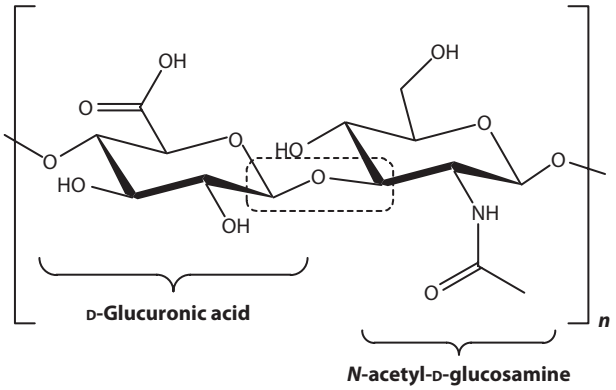


FIGURE 16.18 Disaccharide units of hyaluronic acid linked via β-(1→3).

TABLE 16.6
HPLC Conditions of Glucosaminoglycans

Ref.	Column/Temp.	Mobile Phase	Detection	A: Derivatization B: Guard Column/ Cleanup	Sample
[39]	PGC Hypercarb S 100 × 4.6 mm	Gradient: (A) H_2O –0.05% TFA; (B) AN–0.05% TFA; 0%–100% B	Fluorescence: Ex: 330 nm, Em: 420 nm	A: Precolumn 2-AB B: Ion exchange for cleanup	Glycoproteins, e.g., fetuin
[40]	Anion exchange SS-SAX, 250 × 4.6 mm	Linear gradient: 0.2–1.5 M NaCl	UV 232 nm	A: Sample oxymercuration treatment	Tetra-, hexa-, octasaccharide units of chondroitin sulfate
[40]	APS Hypersil 2 (NH_2), 5 µm, 200 × 4.6 mm	Linear gradient: 100–250 mM NaH_2PO_4, 200–400 mM NaH_2PO_4, 250–450 mM NaH_2PO_4	UV 206 nm	A: Sample oxymercuration treatment	Tri- penta-, heptasaccharide units of chondroitin sulfate
[41]	YMC NH2, 250 × 4 mm, 40°C	Linear gradient of 16–800 mM NaH_2PO_4	UV 210 nm, ESI-MS	B: Cleanup by labeling with ANTS followed by PAGE	Hyaluronan oligosaccharides

Note: 2-AB, 2-aminobenzamide; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; PAGE, polyaclylamide-gel electrophoresis; PGC, porous graphitized carbon.

forms new blood vessels. Owing to the development of HPLC and MS technologies, it has become possible to obtain biologically active HA fragments of defined length from high-molecular-weight HA for angiogenesis.

For analysis, the polymer HA was digested partially, and the enzymatic fragments of HA were labeled with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and then fractionated by SEC on a Bio-Gel P-10 column to give definite oligomers [41]. Fractions resulting from the Bio-Gel P-10 column containing units of disaccharides, probably ranging from the smallest mass of disaccharides to oligosaccharides, were randomly chosen for HPLC analysis to assess their purity. The samples analyzed by HPLC on an NH_2 column using a linear gradient consisting of a NaH_2PO_4–buffer, were further determined by MS to identify their molecular masses (Table 16.6).

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17 HPLC of Plant Lipids

Marek Gołębiowski, Monika Paszkiewicz, Łukasz Haliński, and Piotr Stepnowski

CONTENTS

17.1 INTRODUCTION

17.1.1 Plant Lipids: Definition and Occurrence

The term *lipids* normally covers fatty acids (FAs), their derivatives, and biosynthetically or functionally related substances. But in this chapter it is limited to FAs, their simple derivatives (fatty alcohols [FOHs], aldehydes, hydrocarbons), FA esters (methyl esters [FAMEs] and wax esters [WEs, esters with FOHs]), simple glycerolipids (mono- [MAGs], di- [DAGs], and tri-acylglycerols [TAGs]), complex lipids (phospho- [PLs], sphingo- [SLs], and glycolipids [GLs]), and some lipid biosynthesis intermediates, like acyl-CoA esters. Complex lipids have an additional structural component: PLs contain phosphoric acid in ester form, SLs are founded on sphingoid bases (SBs), usually with an amide-linked FA, and GLs have a sugar linkage [1].

Plant lipids occur as cuticular lipids or waxes on plant surfaces, as internal lipids responsible for membrane integrity and functions, and as an energy source in storage materials like oil seeds. Acting as a barrier between the plant and the environment, the cuticular lipid layer consists mainly of simple FA derivatives and some cyclic compounds [2]. Complex lipids and phytosterols are important cell-membrane components, and seed storage lipids consist almost entirely of simple glycerolipids and minor quantities of FAs.

FAs usually have even numbers of carbon (C) atoms, such as hexadecanoic (palmitic, 16:0) and octadecanoic (stearic, 18:0) acids. This shorthand FA notation gives the numbers of C atoms and double bonds (DBs). It is usually preceded by the DB position and configuration. So c9-18:1 is a monounsaturated FA with 18 C atoms and a *cis* DB between C9 and C10 (C1 is in the carboxylic group). Most plant FAs are polyunsaturated compounds with methylene-interrupted *cis* DBs three C

atoms apart [3]. The most common plant FAs are unsaturated with 18 carbons: c9-18:1 (oleic acid), c9,c12-18:2 (linoleic acid), and c9,c12,c15-18:3 (linolenic acid). Polyunsaturated FAs with DBs on C6 and C9 from the methyl end of the chain (ω-6 and ω-9 acids) are of special interest: They are essential for the human organism and are not synthesized by mammals.

17.1.2 Introduction to Plant Lipids Analysis

High performance liquid chromatography (HPLC), together with thin-layer chromatography (TLC) and column chromatography (CC), are standard methods in lipid class analysis. However, HPLC does not merely separate lipids into classes; single species in each class can also be analyzed. And when HPLC is coupled to a mass spectrometer, the structures of unknown compounds can be determined. Gas chromatography (GC) is often the method of choice for relatively low-molecular-weight FAs and their simple derivatives. Important aspects of plant lipid analysis include determining plant oil composition and the nutritional value of edible plants, and studying lipid biosynthesis and the physiological and ecological roles of plant waxes. In this chapter we review the separation and analysis of standard lipid mixtures and natural plant samples. But rather than attempt to review the entire literature on plant lipid analysis, we present some solutions that are readily applicable to most routine procedures dealing with plant lipids. The following formulas are used for some of the solvents used in lipid extraction and separation: $CHCl_3$, chloroform; CH_2Cl_2, methylene chloride; HCOOH, formic acid; CH_3COOH, acetic acid; and H_2O, water.

Lipids are extracted from tissues with solvents. For neutral lipids relatively nonpolar solvents (hexane, diethyl ether, $CHCl_3$, CH_2Cl_2, toluene) suffice, but for membrane-associated lipids and lipoproteins polar ones (methanol [MeOH], ethanol [EtOH]) are needed to break the lipid-protein hydrogen bonds. $CHCl_3$–MeOH mixtures are used for PLs; GLs are soluble in acetone. Covalent bonds linking lipids to carbohydrates or protein have to be hydrolyzed. Soxhlet extraction is used to remove lipids from solid material. The standard liquid–liquid extraction methods for lipids (all with $CHCl_3$–MeOH) are described in [4–6]. When extracted with $CHCl_3$–MeOH, plant lipids are susceptible to enzyme-catalyzed degradation: The rapid lipase hydrolysis of PLs and GLs yields large amounts of free FAs in the extract. Thus, iPrOH (2-propanol [isopropanol]) is often used to inhibit these enzymes [2]. The plant material is first stirred with iPrOH; then, the mixture is filtered and the solid residue re-extracted with $CHCl_3$–iPrOH (1:1, v/v). If necessary, Folch's procedure [4] is applied to eliminate nonlipid contaminants.

A crude extract of natural lipids usually contains a variety of compounds of different polarities, such as PLs, GLs, and pigments (phenolic derivatives). Due to its complex molecular heterogeneity, several chromatographic steps are needed to separate such a mixture. Traditionally, polar lipid extracts were separated and analyzed by CC and 1D or 2D TLC. Standard TLC plates are glass plates coated with silica gel H or alumina. The silica plate can be coated with magnesium silica or oxalate to improve PL separation [7]; in silver-ion TLC, silica gel G is impregnated with aqueous $AgNO_3$ [8]. Silica gel TLC and Ag-TLC have a polar stationary phase (SP). The major components of the eluent are hexane, petroleum ether, and toluene, while acetone (Ac_2O), diethyl ether, MeOH, EtOH, and CH_3COOH are added in smaller quantities. More polar mobile phases (MPs) containing H_2O or aqueous ammonia will separate complex lipid mixtures. In reversed-phase (RP) TLC the SP is usually bonded octadecyl (ODS, C_{18}) on silica gel, with a mixture of acetonitrile (ACN)–Ac_2O–H_2O as an MP. TLC applications in lipid analysis are reviewed in [9,10].

Column chromatography (Sephadex, ion-exchange cellulose, silicic acid packing; C-18 Sep-Pak cartridges) is used to separate lipid classes of like polarity. With Sephadex columns, the major lipid classes are completely separated from water-soluble nonlipids. Lipids other than gangliosides are eluted first with H_2O-saturated $CHCl_3$–MeOH (19:1, v/v), then gangliosides with $CHCl_3$–MeOH–H_2O containing CH_3COOH, and finally H_2O-soluble nonlipids with MeOH–H_2O

(1:1, v/v) [11]. Silicic acid columns separate neutral lipids from polar ones or GLs with other lipid groups [12]. Ion-exchange columns with DEAE- (diethylaminoethyl) and TEAE- (triethylaminoethyl) cellulose packing provide excellent separation of lipid classes without contamination: The former are more effective for polar lipids, the latter for weakly acidic lipids. Acidic lipids (FAs) are eluted from DEAE columns with weakly acidic solvents, strongly acidic lipids with $CHCl_3$–CH_3COOH–ammonia (3:1:0.1, v/v/v) containing ammonium or potassium acetate. Nonionic lipids are separated on DEAE columns with $CHCl_3$, hexane, or hexane–ethyl ether. The extracts are then eluted using silica gel CC and $CHCl_3$, hexane–diethyl ether (4:1, v/v), hexane–diethyl ether (1:1, v/v), or MeOH and $CHCl_3$–MeOH–H_2O. Neutral lipids, GLs, and PLs are eluted with $CHCl_3$, Ac_2O–MeOH, and MeOH, respectively.

Supercritical fluid extraction has become important: Product solubilities are changed by altering pressure and/or temperature, adding modifiers, or utilizing liquid solvents [13,14]. Packed-column supercritical fluid chromatography with CO_2 and MeOH as MP is recommended as an alternative to liquid chromatography (LC) for lipid class analysis. Lipid classes from a crude wheat lipid extract were completely separated with a 10–40% MeOH gradient in CO_2 [14].

Solid-phase extraction (SPE) for lipid separation has been described, but complete resolution of a complex lipid mixture in a single SPE column is not possible. SPE with silica gel and an aminopropyl-bonded matrix has been used to purify neutral lipids and to recover some PL classes. A single octadecyl-bonded cartridge was used to isolate total phosphatidylcholines, and a single aminopropyl-bonded column employed to separate a wide variety of lipid mixtures [15–17]. Polar lipids can be eluted from a silica gel SP with methyl *tert*-butyl ether (MTBE)–MeOH–ammonium acetate (pH 8.6) [18]. In this case phosphatidylethanolamine and phosphatidylinositol were coeluted in one fraction, but the most polar lipids (phosphatidylcholine, sphingomyelin) were eluted as a second fraction. The major lipid classes (neutral lipids, PLs, nonsialylated SLs, and gangliosides) in their natural states were separated from the same starting material [19]—the method uses solvent mixtures suitable for silicic acid CC and gives a 90–97% recovery for each class. The use of a flexible manifold of three aminopropyl columns to separate different lipid classes was first described in [20]. Serial combination of different SPE columns enhances separation capacity and selectivity [21,22]. A serial manifold of silica gel and aminopropyl-bonded cartridges was used to resolve neutral lipids, GLs, and PLs from wheat flour into individual classes.

Plant membrane lipids (rice and corn leaves, rice leaf stems) were separated by multiple SPE. At the different stages of the glycerolipid separation, the SPE manifold combined aminopropyl, aminopropyl/silica gel, and silica gel/aminopropyl weak anion-exchange columns. The glycerolipid extract was cleared from the pigments onto the aminopropyl column. This was then connected to the silica gel column from which monogalactosyldiacylglycerol, phosphatidylethanolamine, phosphatidylglycerol, and digalactosyldiacylglycerol were eluted as individual fractions. Finally, the silica gel column containing the remaining glycerolipid extract was connected to the aminopropyl anion-exchange column: Individual fractions of sulfoquinovosyldiacylglycerol, phosphatidylcholine, and phosphatidylinositol were eluted.

17.1.3 Detection of Lipids in HPLC Analysis

Many lipid species lack chromophores, so the usual UV detector is unsuitable for this type of analysis. Synthesis of chromophore-containing derivatives does increase UV detector use, but it also complicates the analytical procedure. A fluorescence detector (FD) also requires the synthesis of suitable derivatives. In contrast, a refractometer, though a universal detector, has serious drawbacks: incompatibility with gradient elution and sensitivity to ambient temperature [23,24]. The evaporative light scattering detector (ELSD) has enabled most detection problems to be overcome, at least in lipid class analysis. Detection involves measuring the light scattered by nonvolatile

residual particles after nebulization of the column effluent and MP evaporation, which makes ELSD the universal detector for all substances less volatile than the MP. The MP does not affect results as long as it contains small amounts of nonvolatile residues. The similar charge aerosol detector (CAD), which measures the current from charged residual particles instead of the light scattered, may also be useful. Both ELSD and CAD are destructive detectors, so a line splitter is necessary if the collection of fractions or the use of a different detector in parallel is planned. Rapid advances in HPLC–mass spectrometry (MS) techniques are providing new opportunities in the identification and quantitative analysis of lipids.

All these detectors can be used to analyze lipids quantitatively. But with ELSD there may be a nonlinear response to the amount of substance, sensitivity may be poor [24,25], and calibration curves have to be prepared for each class of compounds to be analyzed. The last drawback also applies to CAD. MS has no such limitations: The intensity of the ions chosen is the basis of quantification.

17.2 LIPID CLASS ANALYSIS

Natural lipid samples usually contain many lipid classes, varying in molecular weight, polarity, and other properties. Analysis basically involves separating the lipid classes from the sample by normal-phase (NP) HPLC and then identifying the sample components in each class with RP-HPLC-MS.

NP-HPLC uses several columns with polar SPs (silica; bonded phases—diol, polymerized vinyl alcohol (PVA), cyanopropyl; alumina) and relatively less polar MPs. A guard column packed with the same material as the main column may increase the latter's lifetime. Simple mixtures of nonpolar lipids can be separated by isocratic elution and any polar column in NP-HPLC. More complex samples are usually separable with silica or diol columns and a binary or ternary MP gradient. Complex mixtures containing simple and polar lipids are separable on silica, diol, or PVA columns, but a ternary gradient of two- or three-component phases is often needed. Depending on the column, the last phase must be polar and contain large amounts of MeOH or H_2O. Separation of nonpolar (simple) and polar (complex) lipids containing FAs on NP-HPLC has been reviewed [26], as have chromatographic techniques for separating and analyzing vegetable oil constituents other than TAGs [27].

Simple lipids are usually separated by NP-HPLC, using ELSD, refractive index (RI) detection, and sometimes UV detection; RP-HPLC in lipid class analysis is far less common. FD and CAD in series with diol and C_{18} columns and a number of gradients were applied to analyze simple plant lipids, tocopherols, and PLs [28], likewise ELSD with silica, diol, cyanopropyl, and alumina SPs [29–34]. A refractometer was used in NP- and RP-HPLC with isocratic elution to separate mixtures of simple lipids [35,36]. UV detection at 295 nm (tocopherols) and 206 nm (unsaturated compounds) is also reported [32,37]. Table 17.1 details the procedures used in these separations; Figure 17.1 and 17.2 illustrate separations of standards and simple plant lipids with a silica SP and ELSD. The example in Figure 17.2 also shows the importance of sample pretreatment when the quantities of lipid classes differ widely.

Separations of mixtures of complex lipids from sterol esters (SEs) to PLs and SLs using gradients (e.g., hexane–iPrOH–water) and silica columns are reviewed in [26]. But these methods will not separate compounds less polar than SEs, so preliminary fractionation may be necessary. A method was developed [38] for the rapid separation of nonpolar lipids, PLs, and SLs using silica columns and ELSD involving a ternary gradient of (A) isooctane–tetrahydrofuran (THF) (99:1), (B) iPrOH–$CHCl_3$ (4:1), and (C) iPrOH–water (1:1) with steps as follows (A/B/C, %): 0–1 min, 100:0:0; 5 min, 80:20:0; 5.1 min, 42:52:6; 20 min, 32:52:16; and 20.1 min, 30:70:0. The analysis was carried out on animal lipids, but it is equally applicable to plant lipids.

Silica-bonded PVA is a useful SP for separating lipids of different polarity. Use of a 5 µm YMC PVA-Sil column (150 × 2 mm) with ELSD to analyze acylglycerols, GLs, and PLs is reported [39].

TABLE 17.1
Examples of Simple Lipid Class Analyses

Sample Type	Stationary and Mobile Phases	Detection	Compounds	Reference
Carnauba wax Grain sorghum wax	Silica, Luna, 5 µm, 250 × 4.6 mm (A) hexane (B) MTBE–CH_3COOH (99.8:0.2) Gradient A/B (%, v/v) (0 min, 100:0; 24–28 minutes, 0:100)	ELSD	FOHs, FAs, TAGs, sterols; WEs, SEs and aldehydes as one peak	[29]
Potato leaf waxes	Silica, 250 × 10 mm (A) hexane (B) CH_2Cl_2–Ac_2O (85:15) (C) CH_2Cl_2–Ac_2O–MeOH (68:12:20) Gradient A/B/C (%, v/v) (0 min, 100:0:0; 20 min, 0:100:0; 30–40 min, 0:0:100)	ELSD	Simple FAs derivatives from hydrocarbons to sterols	[30]
Standards	LiChrospher 100 Diol, 5 µm, 250 × 4 mm (A) heptane (B) heptane–CH_3COOH (100:0.5) (C) heptane–iPrOH–CH_3COOH (50:50:1.5) Gradient (see reference)	ELSD	WE, FAME, FOH, FA, sterol, TAG, DAGs, and MAG	[31]
Corn fiber oil	LiChrosorb Diol, 5 µm, 100 × 3 mm (A) hexane–CH_3COOH (1000:1) (B) iPrOH Gradient A/B (%, v/v) (0–8 min, 100:0; 10–30 minutes, 99.1:0.9)	UV 295 nm ELSD (in series)	FAs, SEs, ferulate esters, TAGs, sterols	[32]
Standards	Alumina (Aluspher), LiChroCART, 5 µm, 125 × 4 mm (A) hexane–THF (99.5:0.5) (B) hexane–THF–iPrOH (60:20:20) Gradient (from 100% to 70% A in 10 min)	ELSD	Hydrocarbons, WEs, SEs, TAGs, sterols	[33]

(*Continued*)

TABLE 17.1 (CONTINUED)
Examples of Simple Lipid Class Analyses

Sample Type	Stationary and Mobile Phases	Detection	Compounds	Reference
Plant oils and products of their transesterification	Cyanopropyl, 250 × 4.6 mm (A) hexane–CH_3COOH (99.6:0.4) (B) MTBE–CH_3COOH (99.6:0.4) Gradient A/B (%, v/v) (0–5 minutes, 100:0; 15–17 min, 20:80)	ELSD	FAs, WEs, MAGs, DAGs, TAGs, sterols	[34]
Green beans (*Phaseolus vulgaris*)	Silica, LiChrosorb Si-60, 5 µm, 250 × 4.6 mm Hexane–iPrOH–CH_3COOH (100:0.14:0.07) Isocratic	RI	FAs, WEs, SEs, TAGs	[35]
Rice bran oil	Hypersil ODS 10, C_{18}, 10 µm, 250 × 4.6 mm Ac_2O–ACN–MeOH (50:50:10) Isocratic	RI	FAs, MAGs, DAGs, TAGs	[36]
Standards	Silica, µPorasil, 300 × 3.9 mm hexane–*n*-butyl chloride–ACN–CH_3COOH (90:10:1.5:0.01) Isocratic	UV 206 nm	FAs, SEs, TAGs, sterols	[37]

Note: Ac_2O, acetone; ACN, acetonitrile; DAG, diacylglycerol; ELSD, evaporative light scattering detector; FA, fatty acid; FAME, fatty acid methyl ester; FOH, fatty alcohol; iPrOH, 2-propanol (isopropanol); MAG, monoacylglycerol; MeOH, methanol; MTBE, methyl *tert*-butyl ether; RI, refractive index detector; SE, steryl ester; TAG, triacylglycerol; THF, tetrahydrofuran; UV, ultraviolet; WE, wax ester.

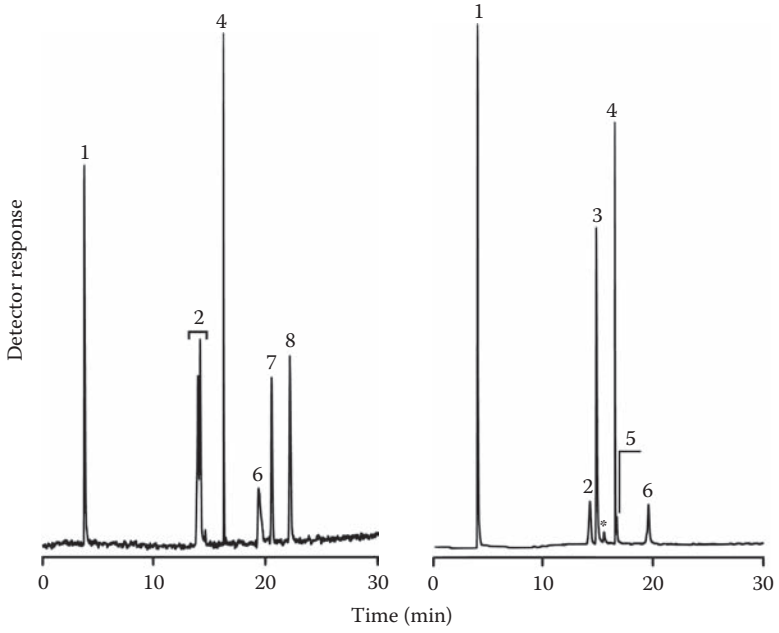

FIGURE 17.1 High performance liquid chromatography–evaporative light scattering detector chromatogram of standards mixture (left) and cuticular waxes of brussels sprout (*Brassica oleracea* var. *gemmifera*, right). Silica column and binary gradient from 100% hexane to 100% methylene chloride-acetone (85:15) were used. 1, hydrocarbons; 2, sterol esters and wax esters; 3, aldehydes; 4, triacylglycerols; 5, ketones; 6, fatty alcohols; 7, free fatty acids; 8, sterols.

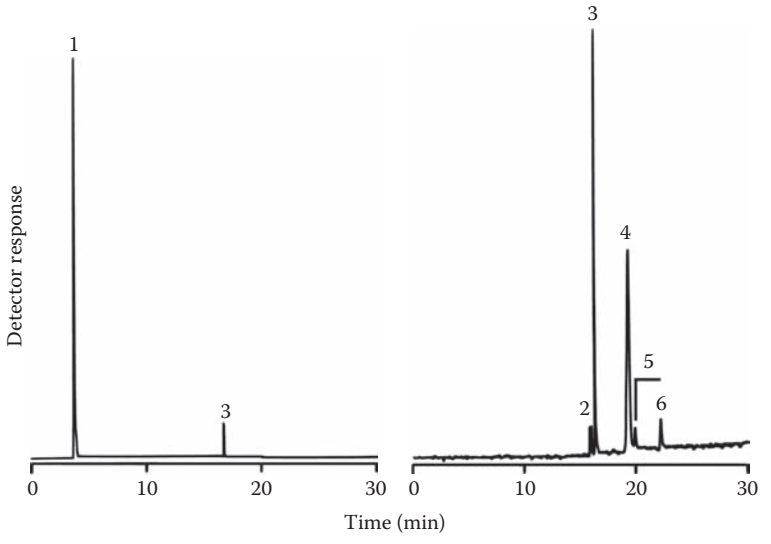

FIGURE 17.2 High performance liquid chromatography–evaporative light scattering detector chromatogram of eggplant (*Solanum melongena*) leaf surface lipids before (left) and after (right) pretreatment using column chromatography on silica gel to remove predominant hydrocarbons. See Figure 17.1 for analytical conditions. 1, hydrocarbons; 2, sterol esters and wax esters; 3, triacylglycerols; 4, fatty alcohols and triterpene alcohols; 5, free fatty acids; 6, sterols.

A binary gradient from heptane–iPrOH (98:2) to $CHCl_3$–iPrOH (65:35) for 25 minutes separated simple lipids, SLs, GLs, and acidic PLs, but neutral PLs and phosphatidylserine were not eluted. A similar gradient, but for 50 min and with a third step using 100% MeOH, separated all the compounds. MPs contained 0.1% triethylamine (TEA)–HCOOH (1:1). The lipid composition of rape (*Brassica napus* L.) seed oil was characterized (ELSD; 250 × 4 mm PVA column; ternary gradient: hexane–MTBE (98:2), iPrOH–ACN–$CHCl_3$–CH_3COOH (84:8:8:0.025), and iPrOH–water–TEA (50:50:0.2) with 1 mM $(NH_4)_2SO_4$ (see Ref. [40]). Polar lipids were analyzed by SPE after preliminary fractionation; lipids from SEs to lysophosphatidylcholine were separated in one step.

17.3 HPLC ANALYSIS OF FATTY ACIDS (FAs)

Free FAs and FAs produced by complex lipid hydrolysis can be analyzed by HPLC. The relevant methods for biological samples and natural, very-long-chain compounds are reviewed in Refs [41,42], respectively. The main problem with the HPLC analysis of single-molecule FAs relates to the detection method. FAs in natural lipids are usually present as mixtures of 12–30 C atom species and unsaturated compounds with different numbers and locations of double bonds. FAs are usually RP-HPLC-separated with octadecyl- (C_{18}) or octyl- (C_8) bonded silica gel and mixtures of H_2O, MeOH, and ACN as MPs [41]. With such phases, detection by ELSD is difficult, and the RI detector is useful for separating simple mixtures only when elution is isocratic. With other detection techniques, suitable derivatives have to be synthesized before analysis. UV-VIS and fluorescence tagging reagents for FA analysis are reviewed in [43]. LC-MS techniques are common in FA analysis: They need no derivatization and guarantee identification and quantitative analysis in one step.

GC is used more often than HPLC to analyze FAs. Nonetheless, HPLC is an interesting alternative for samples with large numbers of different unsaturated compounds, ensuring good separation of single species. FA retention in RP-HPLC depends on C chain length and the DBs, and unsaturated species are eluted ahead of saturated ones with the same chain lengths. The equivalent chain length (ECL) can be calculated for all FAs when difficult-to-separate "critical pairs of compounds" are present. Generally, the retention time of a compound with one DB is similar to that of a saturated FA with two fewer methylene units in the C chain [43]. The presence of more DBs reduces the retention time to a similar degree. Hence, there are groups of compounds with very-similar retention times (16:0, 18:1, 20:2, etc.). Silver-ion chromatography (Ag-HPLC) separates FAs with different numbers of DBs and isomers differing only in DB location. The presence of further functional groups in a FA molecule also affects the retention properties of FFAs and FAMEs [44]. In RP-HPLC C_{18}-keto FAs are eluted before hydroxy FAs, and these before epoxy FAs. *Cis*-isomers are eluted before *trans*-isomers [43,44].

Given the spectroscopic features of RP-HPLC solvents, the preferred UV detection wavelength is ~254 nm. FFAs and FAMEs lack chromophores absorbing in this range, so they are analyzed by ELSD or RI, but UV detection of unsaturated compounds at wavelengths < 215 nm is also possible [44–47]. Table 17.2 lists some examples of FFA and FAME analysis. A rapid (2–6 min) method for separating saturated FAs (C_{16}–C_{26}) using a Purospher Star RP-18e 55 × 2 mm column and triple-quadrupole MS detection with atmospheric pressure chemical ionization (APCI) was developed [48]. MPs were MeOH–H_2O (90:10) and MeOH–hexane (90:10) mixtures with added CH_3COOH. HPLC also serves as a means of sample purification prior to GC analysis. FAMEs were separated from cholesterol using an amino phase column and a gradient of (A) hexane and (B) iPrOH from 98:2 to 90:10 (A/B, %) for 8 min [49].

17.3.1 HPLC Analysis of FA Derivatives

Naphthacyl esters improve the resolution and sensitivity of FA analysis, but they have longer retention times than the corresponding phenacyl derivatives [41,43]. Fluorescence, chemiluminescence, and electrochemical detection can also be applied.

TABLE 17.2
Examples of HPLC Analyses of Free Fatty Acids (FFAs) and Their Methyl Esters (FAMEs)

Sample Type	Stationary and Mobile Phases	Detection	Compounds	Reference
Standards	Ultrasphere ODS, 5 µm, 250 × 4.6 mm Gradients (85–100% MeOH in H_2O in 40 min and then isocratic for FAs, 90%–100% MeOH in H_2O in the same time for FAMEs)	ELSD, UV	49 FAs/FAMEs	[44]
Olive, peanut, palm oils — FAs after TAGs hydrolysis	LiChrospher 100 C_{18}, 5 µm, 250 × 4 mm MeOH–H_2O (89.5:10.5) ACN–H_2O (99:1) Isocratic	RI	FAs C_{16} and C_{18}	[45]
Peanut oil	LiChrospher 100 C_{18}, 5 µm, 250 × 4 mm ACN in H_2O Gradient (80–100% ACN in 10 min and 10 min isocratic)	UV 206 nm	Unsaturated FAs C_{16} and C_{18}	[45]
Soybean, rice bran, and pumpkin seed oils; oil from algae (*Arthrospira platensis*)	Inertsil ODS-3 C_{18}, 5 µm, 250 × 4.6 mm (2 columns in series) MeOH–H_2O (97:3) Isocratic	ELSD	FAMEs C12:0-C22:6	[46]
Microalgae *Scrippsiella* sp.	Alltima C_{18}, 5 µm, 250 × 4.6 mm (analytical) ACN–H_2O (97.5:2.5) isocratic C_{18}, 10 µm, 300 × 22 mm (preparative) Gradient from ACN to $CHCl_3$	ELSD	Polyunsaturated FAMEs C_{16}-C_{28}	[47]

Note: ACN, acetonitrile; ELSD, evaporative light scattering detector; FA, fatty acid; FAME, fatty acid methyl ester; MeOH, methanol; RI, refractive index detector; SE, steryl ester; TAG, triacylglycerol; UV, ultraviolet.

Saturated acids with even numbers of carbons C_{18}–C_{26} from tall oil and tall oil rosin were converted to phenacyl esters using *p*-bromophenacyl bromide solution in ACN (30 min at 77°C), with 18-crown-6 ether as catalyst [50]. After filtration, the mixture was analyzed (250 × 4.6 mm Ultrasphere C_8 column; UV, 254 nm; MP: ACN). Three cyclopropenoid FAs (sterculic, malvalic, and dihydrosterculic acids) from cotton (*Gossypium hirsutum*) seeds and cottonseed oil were converted to phenacyl esters with phenacyl bromide in acetone with TEA (15 min at 100°C and another 5 min after the addition of CH_3COOH). The analysis was performed using a 50 × 4.6 mm C_{18} column and a 150 × 4.6 mm C_8 column coupled in series and UV detection at 242 nm [51]. The MP was ACN–hexane (98:2), isocratic for 34 min, then a gradient to ACN–water (80:20) for 10 min and isocratic for the next 22 min. Twenty-five FFAs (C7:0 to C22:6) as naphthacyl derivatives, synthesized using a solution of naphthacyl bromide in acetone, were separated using a 250 × 4.6 mm C_{18} column and UV detection at 246 nm [52]. A gradient of (A) MeOH, (B) ACN, and (C) water was used with steps as follows (A/B/C, %): 0 min, 80:10:10; 30 min, 86:10:4; 40 min, 90:10:0; and isocratic for 5 min. A mixture of mono- and dicarboxylic acid derivatives was also separated using a gradient of (A) ACN and (B) water (A/B, %): 0–10 min, 50:50; then a gradient to 100:0 for 40 min [52].

Fluorescence detection of FAs is possible after derivatization using fluorescent tagging reagents [43,53]. Saturated C_1–C_{19} FAs from the Tibetan medicinal plant *Lomatogonium rotatum* and C_1–C_{30} FAs from *Gentiana straminea* and *G. dahurica* were identified by LC-MS and quantified using a 150 × 4.6 mm C_8 column and FD after labeling with fluorescent tagging reagents [54,55]. The former FAs were analyzed using a binary ACN–water (20:80) gradient and ACN with respective FD excitation and emission wavelengths of 404 and 440 nm [54], the latter with a quaternary gradient of ACN–water (1:1), ACN–water (1:1) with 0.2 mol dm^{-3} $HCOONH_4$ buffer, ACN–DMF (100:2), and ACN–DMF (100:30), and respective FD excitation and emission wavelengths of 260 and 380 nm [55]. These methods are unique: The FFAs from volatile to long-chain compounds were analyzed in one step.

17.3.2 Methods of Unsaturated FA Analysis

Ag-HPLC is based on the formation of a reversible complex between Ag ions and unsaturated centers in organic molecules, enabling separation of compounds according to the number, position, and geometric configuration of DBs [56]. Ag-HPLC uses two basic types of columns, containing $AgNO_3$-impregnated silica or Ag ions attached to cation exchangers. It analyzes free FAs and FA derivatives (FAMEs, phenacyl esters); aromatic derivatives are resolved better [57]. MPs in Ag-HPLC are like those in other HPLC techniques and should be chosen individually for the FAs to be analyzed and SPs used. Likewise, the detection methods for Ag-HPLC have the same advantages and drawbacks as in other HPLC methods. Ag-HPLC is a specialized technique in lipid analysis and is reviewed in [56,57].

Other chromatographic techniques may be useful for separating unsaturated FAs. Direct analysis of mixtures containing many different compounds does not always ensure sufficient resolution. Ag-TLC was used to separate a mixture of FAMEs into groups according to the number of DBs: Seven fractions (saturated compounds and FAs with 1–6 DBs) were obtained, and simple separation with a C_{18} column and ACN MP fully resolved the mixture [58]. RP-HPLC with two columns in series is a possible alternative [44,59].

17.4 HPLC ANALYSIS OF FA ESTERS AND OTHER SIMPLE FA DERIVATIVES

In the analysis and quality evaluation of plant oils, it is important to quantify the esters, which adversely affect an oil's characteristics. WEs form particulates during the storage of meadowfoam (*Limnanthes alba*) seed oil, used in cosmetic formulations [60]. A simple HPLC method was used to separate and quantify WEs, making up about 0.10% of the refined oil (250 × 4.6 mm silica column; ELSD; isocratic elution with hexane–Ac_2O [95:5] for 5 min). Olive oil quality, too, depends on WE content. WEs

come from the fruit surface, but FAMEs and ethyl esters are products of glycerolipid transesterification with MeOH and EtOH, formed during the fermentation of olives [61]. All WEs were separated in one step (250 × 2 mm silica column; pentane–MTBE [96:4] MP). The fractions were transferred on-line to a GC-MS or GC–flame ionization detector (FID) system to identify and quantify the esters. With this method, FAMEs, ethyl esters, WEs, and SEs could be analyzed simultaneously.

Apple peel wax contained several esters of *Z*- and *E*-*p*-coumaryl alcohols and FAs [62]. Single compounds for structural analysis were isolated by analytical and preparative HPLC (C_{18} 250 × 4.6 mm and 250 × 10 mm columns; RI and UV [258 nm] detectors; MeOH–ACN [4:1] MP). The UV detector response also gave the relative composition of the ester fraction.

Campesteryl, stigmasteryl, and sitosteryl esters with 16:0, 18:0, 18:1, 18:2, and 18:3 FAs were analyzed (HPLC-ELSD and HPLC-MS; 250 × 4.6 mm C_{18} column). Preliminary TLC separation yielded the SE fraction profile of wheat grain species (*Triticum aestivum*, *T. durum*, *T. spelta*, and *T. dicocon*) [63]. A 35%–75% iPrOH gradient in ACN was applied for 35 min in HPLC-ELSD; LC-MS separation was then performed (50%–75% iPrOH in ACN for 25 min, isocratic for the next 75 min, to 90% for 21 min, and isocratic for 10 min). An aqueous solution of ammonium acetate (20 mM dm^{-3}) was added to the LC-MS MPs. HPLC-based methods for analyzing sterols, including steryl esters, are reviewed in [64].

17.5 EXAMPLES OF HPLC ANALYSES IN STUDIES ON PLANT LIPID BIOSYNTHESIS AND METABOLISM

To understand plant lipid biosynthesis and metabolism pathways, it is important to reliably determine intermediates, sometimes at very-low concentrations. For example, HPLC has been used to establish the biosynthetic mechanisms of plant FAs and their derivatives. Acyl-CoA esters from *Arabidopsis thaliana* leaves and seedlings were converted to the corresponding fluorescent acyl-*etheno*-CoA using chloroacetaldehyde and analyzed by RP-HPLC (C_{18} 150 × 2 mm column; quaternary gradient of 1% CH_3COOH, 90% ACN with 1% CH_3COOH, 0.25% TEA with 0.1% THF, and 90% ACN; fluorometer detector—excitation at 230 nm, emission at 420 nm) [65]. FAs were separated with RP-HPLC after incubating plant tissues with radiolabeling agents [66] (gradient of 60–100% ACN in H_2O; MP: 0.01 M H_3PO_4).

Lipoxygenase-pathway-derived aldehydes and their precursors (oxygenated polyunsaturated FAs) in barley (*Hordeum vulgare*) leaves were treated with jasmonate acid methyl ester, then analyzed by NP- and RP-HPLC [67]. The FA derivatives were analyzed by RP-HPLC (C_{18} 250 × 4 mm column; MP: MeOH–H_2O–CH_3COOH [85:15:0.1]; UV detection). Aldehydes and hydroxy aldehydes after derivatization with 2,4-dinitrophenylhydrazine were analyzed in a similar way but with ACN and H_2O gradients. Hydroxylinoleic acid isomers were analyzed by NP-HPLC (silica 250 × 4.6 mm column; MP: hexane–iPrOH–CH_3COOH [100:2:0.1]). The enantiomeric composition of hydroxy FAs was also studied by chiral-phase HPLC. Hydroxy FAs and FA-derived aldehydes from wounded cucumber plants (*Cucumis sativus*) were analyzed in a similar manner, but the aldehydes were separated by isocratic elution with ACN–THF–water (74:1:25) instead of ACN–water [68].

17.6 HPLC OF SIMPLE GLYCEROLIPIDS

Simple glycerolipids (MAGs, DAGs, and TAGs) are often the most abundant components of vegetable oils and animal fats [27,69]. Acylglycerols are synthesized by the esterification of glycerol with saturated and unsaturated FAs. Naturally occurring TAGs differ in acyl chain length but also in the number, location, and *cis*/*trans* configuration of DBs in each chain. Positional isomerism and *R*/*S* optical isomerism also occurs, the latter in TAGs containing differing acyl chains, at least in positions 1 and 3 (asymmetrical TAGs) [70,71].

Interest in HPLC for TAG determination has much increased, the focus being on the most common techniques—Ag-HPLC [72–75] and nonaqueous reversed-phase (NARP)-HPLC [69,70,76].

GC [70] and TLC [77] have also been used, as have some less common techniques like capillary electrochromatography (CEC) [78], supercritical fluid chromatography [79], and subcritical fluid chromatography [80]. The usefulness of HPLC is extended by the much greater choice of MPs in each mode (NP-HPLC, NARP-HPLC, Ag-HPLC) than is possible with other chromatographic methods. Ag-HPLC separates TAGs differing in the number and position of DBs (the more DBs, the better the TAG retention). NARP-HPLC also separates TAGs differing in acyl chain length and the number and position of DBs. Here, the retention time increases with equivalent carbon number (ECN)—the difference between the total number of C atoms and twice the number of DBs in all acyl chains. Under optimal conditions, compounds with the same ECNs can be separated [76].

The drawbacks of the detection methods used in HPLC analysis of lipids have already been outlined; they also apply to acylglycerol analysis. UV detection, commonly used in HPLC, is impossible with UV wavelengths >220 nm—TAGs do not absorb them [71,76]. Common TAGs are detected by low-wavelength UV (205–210 nm), which gives linear calibration curves and adequate sensitivity even with gradient elution. With saturated TAGs, however, UV detection sensitivity is low, which precludes their quantitative analysis. Other detectors (e.g., ELSD, CAD, RI) are widely used in TAG analysis. Several HPLC-MS-based methods have also been reported.

RI detection in tandem with isocratic elution is limited to mixtures of simple TAGs [81]. In contrast, universal ELSD and CAD can be used together with gradient elution and are thus more suitable for separating mixtures of complex TAGs. However, the CAD response depends on the amount of organic solvent in the MP [82]. ELSD has been applied in TAG analysis by HPLC [76,83–85]. The mass spectrometer is a possible alternative to classical detection methods: It permits rapid separation and quantification of TAGs, with simultaneous identification of single species on the basis of the mass spectra. MS is also much more sensitive than chromatographic detectors. TAGs have been analyzed by HPLC-MS using a quadrupole mass spectrometer and soft ionization techniques like electrospray ionization (ESI) and APCI. APCI is the standard technique for TAG analysis because of its high ionization efficiency and full compatibility with nonaqueous MP systems. Single TAGs are identified on the basis of characteristic ions (protonated molecules $[M+H]^+$ and fragment ions $[M+H\text{-}R\textit{i}COOH]^+$) [86,87]. ESI yields $[M+Na]^+$ and $[M+K]^+$ ions, and some fragment ions like $[M+Na\text{-}R\textit{i}COOH]^+$ and $[M+Na\text{-}R\textit{i}COONa]^+$, which usually have lower-relative abundances [70]. Table 17.3 briefly reviews the analytical methods used in TAG separation.

MP composition is the main factor affecting TAG separation by RP-HPLC, as it uses only a very small number of SPs. As TAGs are H_2O-insoluble, apolar solvents are usually used in their analysis. Various MPs, mostly in gradient elution mode, are used to separate TAGs. Typical solvent systems in RP-HPLC consist of ACN, iPrOH, hexane, Ac_2O, $CHCl_3$, CH_2Cl_2, and their mixtures [70,76,82]; toluene, CH_2Cl_2, ethylene chloride, hexane, ethyl acetate, and their mixtures are common MPs in Ag-HPLC [72,88].

MAGs and DAGs are determined and quantified using the same separation techniques as for TAGs (RP-HPLC, Ag-HPLC, and HPLC-MS) [71,89,90]. HPLC methods using different SPs, solvent mixtures, and modes of detection are therefore available for the complete analysis of simple glycerolipids.

17.7 HPLC ANALYSIS OF PHOSPHOLIPIDS (PLs)

Lipid extracts from plant sources contain a wide variety of complex compounds, including PLs, GLs, and SLs. PLs contain phosphoric residues, polar head groups, and nonpolar lipid chains. Thus, both glycerolphospholipids and phosphosphingolipids can be referred to as PLs. Lipids containing phosphoric acid ester are common in animal tissues, plants, and plant seed oils and are important components of biological membranes [91,92], hence the numerous publications on PL separation and analysis.

PL levels in natural samples are low, so they need to be concentrated before analysis. The PL fraction can be isolated from other lipid classes in various ways, including TLC, CC, and SPE

TABLE 17.3
Examples of TAG Separations Using Various HPLC Systems

Sample Type	Mobile Phase	Detection	Stationary Phase	Reference
Standards	MeOH–iPrOH, MeOH–$CHCl_3$, MeOH–CH_2Cl_2, ACN–iPrOH, ACN–$CHCl_3$, ACN–CH_2Cl_2 Binary gradients (see reference)	APPI-MS	Gemini C_{18}, 5 µm, 150 × 2 mm	[69]
Conifer seed oils: *Larix decidua*, *Picea abies*, *Abies alba*	(A) ACN (B) iPrOH Gradient A/B (%) (0 min, 100:0; 106 min, 31:69; 109 min, 100:0)	APCI-MS UV 205 nm	Nova-Pak C_{18}, 4 µm, 300 × 3.9 mm and 150 × 3.9 mm (in series)	[70]
Plant oils, including walnut, hazelnut, almond, and fig oils	(A) ACN (B) iPrOH Gradient A/B (%) (0 min, 100:0; 106 min, 31:69; 109 min, 100:0)	APCI-MS UV 205 nm ELSD	Nova-Pak C_{18}, 4 µm, 300 × 3.9 mm and 150 × 3.9 mm (in series)	[76]
Vitis vinifera	ACN–Ac_2O–THF (58:38:4) Isocratic	RI	Sugelabor Spherisorb ODS, 5 µm, 200 × 4.6 mm	[81]
Plant oils	(A) hexane–iPrOH (1:1) (B) ACN Gradient A/B (%) (0 min, 20:80; 80 min, 75:25)	CAD	Hypersil ODS, 5 µm, 250 × 4.6 mm (two columns in series)	[82]
Walnut (*Juglans regia*) cultivars	Ac_2O–ACN (70:30) Isocratic	ELSD	Kromasil 100 C_{18}, 5 µm, 250 × 4.6 mm	[85]
Plant oils, including palm, olive, rapeseed, soybean, and corn oils	(A) ACN (B) iPrOH Gradient A/B (%) (0 min, 100:0; 106 min, 31:69; 109 min, 100:0)	APCI-MS	Nova-Pak C_{18}, 4 µm, 300 × 3.9 mm and 150 × 3.9 mm (in series)	[86]
Hazelnut and olive oils	Ac_2O–ACN (64:36) Isocratic	APCI-MS	Spherisorb ODS2, 5µm, 250 × 4.6 mm	[87]

Note: Ac_2O, acetone; ACN, acetonitrile; APCI-MS, atmospheric pressure chemical ionization–mass spectrometry; APPI-MS, atmospheric pressure photoionization–mass spectrometry; CAD, charge aerosol detector; ELSD, evaporative light scattering detector; iPrOH, 2-propanol (isopropanol); MeOH, methanol; RI, refractive index detector; TAG, triacylglycerol; THF, tetrahydrofuran; UV, ultraviolet.

[93,94]. HPLC is often the method of choice because of its simplicity and sensitivity. HPLC separation of PLs containing primary amino groups was first done in 1975 [95]; now there are many HPLC methods for PL analysis using different columns, MPs, and detectors. Typically, PLs are separated by NP-HPLC, usually with a silica gel SP [93,96]: PLs elute in order from the least polar to the most polar species. Molecular PL species are separable by RP-HPLC and NP-HPLC using amino and diol SPs. With RP-HPLC, compounds can be separated according to the degree of FA unsaturation [93].

Full PL separation in one isocratic run is difficult, if not impossible, so gradient elution is usually applied. To determine the separation conditions for such an analysis, gradient time and MP composition need to be adjusted. The following solvent systems are routinely applied in PL separations: hexane–iPrOH–water, ACN–MeOH–water, and $CHCl_3$–MeOH–water [97].

The detection mode is a serious limitation in HPLC analysis of PLs: Their lack of chromophores makes their UV quantification difficult. But the carbonyl and phosphate groups in PLs facilitate their analysis in that absorption if the 200 nm region is measured. MPs consisting of hexane, iPrOH, ACN, and MeOH, which do not absorb appreciably in the 190–210 nm UV range, have been used successfully in NP- and RP-HPLC separations. Other detectors are also widely used: ELSD [94,98], MS [99,100], UV [92,96,101], and FID [102]. Table 17.4 lists HPLC methods for PL analysis.

The sensitivity, selectivity, and specificity of HPLC-MS methods makes them the most convenient analytical techniques in PL analysis. LC-MS combined with ESI leads to reproducible identification and quantitative analysis of PLs [99]. LC-ESI-MS distinguishes PL classes and identifies individual molecular species by collision-induced decomposition pathways. As simultaneous quantification is possible, this is the method of choice to characterize the molecular compositions of PLs in detail. Direct analysis of lipid extracts by ESI-MS/MS without previous chromatographic separation (loop injection) is also possible, determining the structure of individual species in a single analysis. Tandem MS/MS (without prior HPLC separation) for quantifying the most common PL classes employs triple-quadrupole or quadrupole time-of-flight (QTOF) mass spectrometers. The importance of HPLC-MS methods in PL analysis may increase in the next few years.

Other techniques, like 1D or 2D TLC [97], subcritical fluid chromatography [91], nonaqueous capillary electrophoresis (NACE) [103], micellar electrokinetic chromatography (MEKC) [104], matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [105] have been adapted for PL analysis. Another useful approach is ^{31}P nuclear magnetic resonance (NMR) spectroscopy, but the prior application of a preparative-scale separation technique like HPLC is needed [106].

17.8 HPLC ANALYSIS OF GLYCOLIPIDS (GLs)

GLs are a diverse class of heterogeneous conjugates composed of sugars and lipids found in organisms from bacteria to mammals [107]. They contain one or more monosaccharide residues linked via a glycoside to a hydrophobic moiety such as an acylglycerol, sphingoid, ceramide, or prenyl phosphate. They are generally classified as glycoglycerolipids, glycosphingolipids, and glycophosphatidyl inositols. The major class of plant GLs is the glycosyl diacylglycerols. Glycoglycerolipids exhibit antiviral [108], antitumor [109–111], and anti-inflammatory [112] properties. They also appear to inhibit [113] and promote [114,115] cell growth and afford protection against cell death [109,111,116].

Glycosyl diacylglycerols are the major building blocks of biological membranes. Membrane composition in plant chloroplasts and cyanobacteria is highly conserved, with monogalactosyldiacylglycerols (MGDGs) and digalactosyldiacylglycerols (DGDGs) being the most abundant lipids (approximately 50% of GLs consist of MGDGs) [117]. Other thylakoid lipids include the acidic sulfoglycolipids (sulfoquinovosyldiacylloglicerols, SQDGs). The galactose residue in MGDGs is bound to the *sn*-3 position of the glycerol backbone in the β-anomeric linkage. The DGDG head group contains a terminal α-galactose moiety (1→6) linked to the inner Gal residue. Some plants (e.g., adzuki

TABLE 17.4
Examples of HPLC Analyses of PLs

Sample Type	Mobile Phase	Detection	Stationary Phase	Reference
Leaves of soybean (*Glycine max*)	Isooctane–iPrOH–H_2O (40:53:7) Isocratic	UV 205 nm	Excello silica, 5 µm, 150 × 10 mm	[93]
Leaves of soybean (*Glycine max*)	MeOH–0.1 M ammonium acetate (95:5) Isocratic	UV 205 nm	Excello Ultra Pac ODS, 5 µm, 150 × 4.6 mm	[93]
Wheat flour	(A) hexane–iPrOH–CH_3COOH–TEA (81.42:17.00:1.50:0.08) (B) iPrOH–H_2O–CH_3COOH–TEA (84.42:14.00:1.50:0.08) Gradient (see reference)	ELSD	LiChrospher 100 Diol, 5 µm, 100 × 4 mm	[94]
Corn oil	(A) hexane–iPrOH–CH_3COOH–TEA (81:17:1:0.08) (B) iPrOH–H_2O–CH_3COOH–TEA (85:14:1:0.08) Gradient A/B (%) (0 min, 95:5, 25–26 min, 20:80, 36–40 min, 95:5)	ESI-MS	LiChrospher 100 diol, 5 µm, 250 × 4.6 mm	[99]
Olive and sunflower oils	(A) ACN–MeOH (B) MeOH (C) H_2O Gradient (see reference)	UV 208 nm	Sphereclone, 5 µm, 250 × 4.6 mm	[101]
Spinacea oleracea, *Arabidopsis thaliana*	ACN–MeOH–CH_3COOH–H_2O–1-ethylpropylamine (89.8:6.8:1.5:1.0:0.9) Isocratic	FID	Rainim Microsorb, 5 µm, 250 × 4.6 mm	[102]

Note: ACN, acetonitrile; ELSD, evaporative light scattering detector; ESI-MS, electrospray ionization–mass spectrometry; FID, flame ionization detector; iPrOH, 2-propanol (isopropanol); MeOH, methanol; PL, phospholipid; RI, refractive index detector; TEA, triethylamine; THF, tetrahydrofuran; UV, ultraviolet.

bean, *Vigna angularis*) contain additional DGDGs formed from two Gal residues in the β-anomeric configuration. In contrast to the conserved head group, galactolipid FAs are highly variable. Each of the two major galactolipid classes, MGDGs and DGDGs, consists of various molecular species differing in chain length, degree of unsaturation, and fatty acyl group distribution.

The amphipathic nature of GLs means that special techniques are needed for their separation: The major ones are TLC and HPLC [118]. The profiles of carbohydrates and lipids are determined and their structures analyzed by chemical methods. These are used in conjunction with GC and HPLC, sometimes coupled to MS. With enzymatic methods the linkage sequence and anomeric configurations of oligosaccharide structures can be determined and the position of the FA in diacylglycerol species established. MS and NMR are invariably used to determine the full structural features of an unknown GL. HPLC is the most efficient way of separating and estimating the amount of each glycoglycerolipid in an unknown sample. A UV detector is not very effective for sugars, which lack specific UV-absorbing chromophores. While sugars absorb appreciable amounts of low-wavelength UV, the chromophores are relatively weak due to the poor selectivity over many of the other components [119]. However, some methods of GL analysis using low-wavelength UV detection have been developed [120–124]. ELSD allows direct quantification of lipid compounds without interference from MP components, as demonstrated by UV detection. ELSD analyses are reported [121,125–129]. The RI detector is also useful in carbohydrate analysis [123,130,131]. Table 17.5 lists examples of HPLC analyses of GLs.

The first use of HPLC to extract GLs from plant matter enabled all the polar lipids to be separated from cereal grains [126]. A complex ternary solvent gradient system separated both GLs and all the lipid classes in wheat flour [127]. GLs from edible plant sources were accurately quantified by silica-based NP-HPLC using an ELSD [128]. Five major GL classes (acylated steryl glucosides, steryl glucosides, ceramide monohexosides, MGDGs, and DGDGs) were separated and determined with a binary gradient system consisting of $CHCl_3$ and MeOH–water (95:5) without interference from other lipid classes and pigments. This method was applied to 48 edible plants available in Japan, including cereals, legumes, fruits, and vegetables. MGDGs, DGDGs, and SQDGs were quantified with a method for the simultaneous quantitative analysis of neutral and acidic lipids using HPLC-ELSD [129]. Ten complex lipid classes were separated in 16 min using a binary gradient system of $CHCl_3$ and MeOH–Ac_2O–H_2O–CH_3COOH (30:60:9:1) with 0.3% TEA (pH 4).

With HPLC-MS, GL species can be separated and their structures determined; the importance of such techniques is thus beyond doubt [120,122,132,133]. Chromatographic separation of polar lipid classes with NP-HPLC was achieved in only 18 min, and the molecular species were characterized by ESI(+)-MS/MS [132]. The HPLC retention time increased in the order MGDGs < glycocerebrosides < DGDGs < PLs. The positive ionization mode was more suitable than the negative for simultaneous GL determination [132]. Molecular species of MGDGs and DGDGs were detected as their adducts with ammonium $[M+NH_4]^+$, GCers as pseudomolecular ions $[M+H]^+$. The product ion spectrum from MS/MS of MGDGs and DGDGs yielded fragments due to the loss of an acyl group $[M-RCO+2H]^+$ and the neutral loss of the glycosyl moiety. The determination of fatty acyl groups of molecular species present in complex mixtures of MGDGs and DGDGs using HPLC-ESI-MS combined with in-source fragmentation in the negative-ion mode is reported [134]. In this mode, all classes of galactolipids have a similar fragmentation pattern. The major ions produced by the cleavage of acyl moieties provide information about the FA composition of each component. Ions were also obtained as a result of eliminating $RCOO^-$ from the $[M-H]^-$ ion. In addition to providing information on the composition of the two acyl groups, negative-ion in-source fragmentation enables the polar head group to be identified from the difference between the molecular masses of the two FAs and the total molecular mass. Sixteen new and 10 known galactolipids have been isolated and determined from the leaves of *Ipomea batatas* L. using HPLC + ESI-QTOF-MS/MS [128]. The structures of the fatty acyl groups and the positions of the DBs on the acyl chains were thus determined. Individual galactolipids were eluted isocratically (C_{18} column; MP: MeOH–water–ACN

TABLE 17.5
Examples of HPLC Analyses of GLs

Sample Type	Stationary and Mobile Phases	Detection	Compounds	Reference
Leaves of *Ipomoea batatas*	Hypersil BDS C_{18}, 250 × 4.6 mm, MeOH–H_2O–ACN (90.5:7:2.5) MeOH–H_2O–ACN (82.5:15:2.5) Isocratic	UV 205 nm ESI-MS/MS	MGDGs, DGDGs	[120]
Leaves of *Byrsonima crassifolia*	C_{18} μ-Bondapak, 300 × 7.8 mm, MeOH–H_2O (90:10) Isocratic	ELSD, UV	Four new glycolipids	[121]
Glycolipids from pea leaf photosystem I complex	Bondapak C_{18} 10μm, 3.9 × 300 mm, (A) H_2O (B) ACN Gradient A/B (%) (0–5 min, 95:5; 13 min, 35:65; 20–30 min, 5:95)	UV 205 nm	MGDGs, DGDGs, SQDGs	[122]
Clove, red pepper, and nutmeg	Aquasil-SS, 200 × 6 mm, (A) iPrOH (B) *n*-hexane (C) H_2O Gradient A/B/C (%) (0 min, 20:80:0; 20–30 min, 55:40:5)	UV 208 nm	Neutral glycosphingolipids	[123]
Clove, red pepper, and nutmeg	C_8, 250 × 4.6 mm MeOH–H_2O (96:4) Isocratic	UV 205 nm	Glycoglycerolipids	[123]
Clove	Aquasil-SS, 200 × 6 mm, iPrOH–*n*-hexane–H_2O (55:40:5) Isocratic	RI	Neutral glycosphingolipids	[123]
Black cumin, coriander, and niger oilseeds	Zorbax-Sil (5 μm) isooctane–iPrOH (1:1) Isocratic	UV 206 nm	MGDGs, DGDGs, SQDGs, steryl glucosides	[124]

(*Continued*)

TABLE 17.5 (CONTINUED)
Examples of HPLC Analyses of GLs

Sample Type	Stationary and Mobile Phases	Detection	Compounds	Reference
Apple and carrot tissues	ChromSep LiChrosorb Si 60, 100 × 3 mm, (A) iPrOH (B) hexane (C) H_2O Gradient (see reference)	ELSD	MGDGs, DGDGs, steryl glycosides	[125]
Pulp and almond of the avocado fruit (*Persea americana*)	Polaris Si-A 3µ,150 × 4.6 mm, $CHCl_3$–MeOH–H_2O–NH_4OH (30%): (A) (80:19.5:0:0.5) (B) (60:34:5.5:0.5) Gradient A/B (%) (0 min, 100:0; 10–25 min, 0:100)	ESI-MS	MGDGs, DGDGs	[132]

Note: ACN, acetonitrile; DGDG, digalactosyldiacylglycerol; ELSD, evaporative light scattering detector; ESI-MS, electrospray ionization–mass spectrometry; GL, glycolipid; iPrOH, 2-propanol (isopropanol); MeOH, methanol; MGDG, monogalactosyldiacylglycerol; RI, refractive index detector; SQDG, sulfoquinovosyldiacyloglicerol; UV, ultraviolet.

[90.5:7:2.5]). Separation of more polar galactolipids was improved with an MP of MeOH–water–ACN (82.5:15:2.5).

17.9 HPLC ANALYSIS OF SPHINGOLIPIDS (SLs)

SLs are a class of complex membrane lipids found in all eukaryotic and some prokaryotic cells, where they contribute to membrane structures and are involved in the regulation of cell metabolism [133,135]. In contrast to the more prevalent glycerolipids (such as PLs or galactolipids), which have two fatty acyl chains and a polar head group linked to a glycerol backbone complex, SLs consist of a sphingoid long-chain base (amino alcohol) with one fatty acyl chain and a polar head group [136]. Higher plants and fungi contain a group of SLs not found in animals, with a ceramide (CER) backbone consisting of a C_{18} long-chain base, usually hydroxysphinganine (phytosphingosine). CER backbones of plant SLs can be acylated by more than 10 different FAs. These are almost exclusively α-D-hydroxylated and vary in chain length from C_{14} to C_{26}, including significant, but not predominant, chains with odd numbers of carbons [133]. Saturated C_{16}, C_{20}, C_{22}, and C_{24} α-hydroxylated FAs are the most common, whereas ω-9-monounsaturated, very-long-chain FAs ranging from C_{22} to C_{26} occur in low-proportions. The CER is substituted at its terminal hydroxyl group with polar residues like carbohydrate, phosphoinositol, and phosphocholine to form complex SLs. The major complex SLs identified in plants are monoglucosylceramides (glucocerebrosides), containing a glucose head group, and the more polar inositol phosphate-based SLs, collectively referred to as inositolphosphoceramides [137,138]. The diversity, biosynthesis, and functions of plant SLs are reviewed in [139].

The differences in head groups and alkyl chain lengths provide distinct chromatographic and MS characteristics of SLs. It is important to separate and quantify SLs for study of their concentrations and biological functions. But it is hard to isolate SLs, particularly free CERs, as they typically make up < 1% of the total extractable lipids [140]. Different approaches exist to identify and quantify SLs, such as TLC [141], HPLC [142], and MS. Some MS methods, especially ESI-MS/MS combined with LC, facilitate molecular species analysis [143–146].

Free CERs were determined with HPLC and UV detection [147]. CERs were derivatized with benzoyl chloride or benzoyl anhydride to give them a UV-absorptive tag at 230–280 nm, enabling them to be quantified. Despite the good quantitative results, this procedure is time-consuming, the key reaction is very water-sensitive, and all reagents have to be prepared fresh each time and handled anhydrously. CER derivatives are unstable, requiring immediate analysis. CERs were separated directly and analyzed structurally by HPLC after derivatization with benzoyl and *p*-nitrobenzoyl chloride. The structures of the derivatives of the CER *N*-heptadecanoyl D-sphingosine were MS-characterized. Molecular CER species (the acyl group and the long-chain base) were separated using a 10 µm LiChrosorb C_{18} column and isocratic elution [148].

A method for the quantitative analysis of molecular CER species by RP-HPLC + FD involved CER species separation according to FA chain length and the long-chain base backbone [149]. It is useful for elucidating the functions of CER subspecies. Several methods of quantifying SL with HPLC-ELSD are reported [150,151]. CER and SBs—phytosphingosine, dihydrosphingosine, sphingosine, and sphingosine 1-phosphate—were separated by NP-HPLC and detected with ELSD [152]; they were resolved with a rapid and quantitative assay in the nM range. Phytosphingosine was resolved as a sharp peak with TEA and HCOOH added to a $CHCl_3$–EtOH MP. CER-SB resolution was acceptable, but the peaks of DN (dihydroxysphingosine with normal FAs) and DA (dihydroxysphingosine with R-hydroxyl FAs) overlapped. DN was effectively separated from DA on a cyanopropyl-bonded column without derivatization, but separation of DA from TA (trihydroxysphingenine with R-hydroxyl FAs) and DN from TN (trihydroxysphingenine with normal FAs) was not possible [153]. Four CERs were quite well resolved with both HPLC-ELSD and HPLC-APCI-MS, although one of the two TA peaks still overlapped the TN peak [154].

HPLC-ELSD methods for analyzing glucosylceramides (GlcCer) and CER in soybeans are described in [155,156]. The MP A was hexane–THF (99:1), while the MP B was changed to

TABLE 17.6
Examples of HPLC Analyses of SLs

Sample Type	Stationary and Mobile Phases	Detection	Compounds	Reference
Arabidopsis thaliana leaves	Xpertek Spherisorb silica amino 250 × 4.6 mm (A) iPrOH–hexane–H_2O–CH_3COOH[a] (60:35:4:1) (B) iPrOH–H_2O–CH_3COOH[a] (60:39:1) Gradient A/B (%) (0–5 min, 100:0; 10–20 min, 75:25; 30 min, 0:100)	ESI-MS	Anionic sphingolipids	144
Arabidopsis thaliana leaves	Xpertek Spherisorb silica-amino 250 × 4.6 mm (A) ACN–MeOH–CH_3COOH (97:2:1)[a] (B) MeOH–CH_3COOH (99:1)[a] Gradient A/B (%) (0–3 min, 100:0; 23 min, 0:100)	ESI-MS	Neutral sphingolipids	144
Soybean (15 lines)	PhaseSep silica 150 × 4.6 mm $CHCl_3$–EtOH–TEA–HCOOH (90:10:1:1) Diol 150 × 4.6 mm $CHCl_3$–EtOH–TEA–HCOOH (80:20:1:1) Isocratic	ELSD	Ceramides, GlcCer	155
Standards	Diol (A) hexane–iPrOH–CH_3COOH (82:17:1) (B) iPrOH–H_2O–CH_3COOH (85:14:1) 0.08 % TEA added to both phases Gradient (see reference)	LLSD	D-*erythro*-(2*S*,3*R*)-, D-*threo*-sphingomyelin	158
Standards	Supelco Discovery C_{18} 50 × 2.1 mm (A) MeOH–H_2O–HCOOH (58:41:1)[b] (B) MeOH–HCOOH (99:1)[b] Gradient A/B (%) (0–0.5 min, 60:40; 1.8–5.3 min, 0:100)	MS	Sphingoid bases, sphingoid base 1-phosphates, and ceramide 1-phosphates	162
Standards	Supelco LC-Si, silica 250 × 2.1 mm ACN–MeOH–CH_3COOH (97:2:1)[a] Isocratic	MS	GlcCer, GalCer	162

Note: ACN, acetonitrile; ELSD, evaporative light scattering detector; ESI-MS, electrospray ionization–mass spectrometry; EtOH, ethanol; iPrOH, 2-propanol (isopropanol); MeOH, methanol; MS, mass spectrometry; SL, sphingolipid; TEA, triethylamine; LLSD, laser light scattering detector.

[a] Phase contains 5 mM ammonium acetate;

[b] Phase contains 5 mM ammonium formate.

MeOH–MTBE (75:25). The method gave an acceptable separation of DN and DA when 1% HCOOH was added to solvent B, but the resolutions of DA, TA, and TN were poor. These CERs were finally separated with a gradient of hexane and iPrOH–ethyl acetate–88% HCOOH (50:50:0.5), the elution order being DN, TA-1, TN, DA, ESG, and TA-2 (TA contained racemic isomers of the R-OH FA).

A method for separating molecular CER species by Ag-HPLC based on the number of DBs regardless of the number of C atoms are described. The 3-*O*-benzoylated nonhydroxy FA containing CERs was resolved (Ag column; MP: hexane–iPrOH [9:1]); resolution of minor peaks was better with a 9.5:0.5 ratio of the same solvent. Hydroxy FA-containing CERs were separated without derivatization by Ag-HPLC with hexane–iPrOH (9:1) [157].

The separation of naturally occurring sphingomyelin diastereomers is reported [158]—these all have the D-*erythro*-(2*S*,3*R*) configuration of the sphingoid base. An NP-HPLC method was developed for separating such a stereoisomer from D-*threo*-sphingomyelin on a diol column using a binary gradient of hexane–iPrOH–CH_3COOH (82:17:1.0) and iPrOH–water–CH_3COOH (85:14:1.0) with 0.08% TEA added to both mixtures. Lipids were detected by MS. ESI-MS/MS can quantify different CER species from crude cellular lipid extracts without prior separation [159]. Li^+ cationization in conjunction with ESI-MS and low-energy collision-induced dissociation mass spectrometry (CIDMS) was very effective for the detailed structural analysis of complex fungal cerebrosides (monohexosylceramides) [160]. SL molecular species and their compositions were directly determined using HPLC-MS/MS and simple loop injections and multiple reaction monitoring (MRM) [161]. Alternatively, HPLC-MS/MS methods were used, and SLs were eluted by classes. Each individually optimized MRM method could thus be used at specific times in a single HPLC run, providing a quick analytical method for all SLs with highly sensitive and accurate quantitation. A number of other MS-based methods for PL standards have been reported [162]. Table 17.6 lists several HPLC-based methods of SL analysis.

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18 HPLC Analysis of Amino Acids, Peptides, and Proteins

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CONTENTS

18.1 INTRODUCTION

Proteins are an important fraction in most foodstuffs, ranging from 1.8% in roots and tubers up to about 25% in legumes. However, in foods they are present in a complex multicomponent matrix also containing water, lipids, carbohydrates, and some minor components, all of which might interfere during the analysis of proteins in foods. A whole variety of analytical tools for protein analysis are available to the analyst, but not all of them are suitable for routine analysis procedures. Well-established methods are the Kjeldahl method; spectroscopy in the ultraviolet (UV), visible (VIS), and infrared (IR) ranges of the spectrum; conventional electrophoresis; capillary electrophoresis (CE); immunoassay; and chromatography.

In recent times high performance liquid chromatography (HPLC) has emerged as a method of choice for analytical purposes regarding plant protein evaluation. The biggest advantage that it has over other techniques is the speed of analysis, which is many times higher than that of other techniques except, perhaps, for gas–liquid chromatography (GLC). The sample requirement is also very low for this technique and as little as a few femtograms of the sample will be analyzed satisfactorily. On top of all this, the detectors that are employed in HPLC are nondestructive in nature, and thus the separated components can be recovered for further study. HPLC has been successfully applied to the separation of proteins, nucleic acids, polysaccharides, plant pigments, amino acids, pesticides, medicines and their metabolites, animal and plant hormones, and complex lipids.

This chapter deals with some HPLC analytical techniques available for protein analysis of plant extracts in order to give an overview of possibilities and shortcomings of these techniques and some

promising future trends. Since validation of methods and results are important features of today's analysis, some attention is paid to the availability and use of reference materials.

However, if anything has promised to completely transform plant protein analysis, it is the development and use of multidimensional HPLC as a replacement for classical bidimensional electrophoresis (2DE). The potential benefits of using HPLC rather than 2DE are immense. The most prominent are:

- No long gel runs
- No gel removal steps or gel interactions
- Fewer size limitations (HPLC can accommodate molecules ranging from amino acids and/or small peptides to large proteins)
- No visualization problems with regard to the need for staining
- Greater sensitivity
- The ability to act as an immediate portal to subsequent HPLC runs
- Multiplicity of kinds of HPLC separation possible
- The ability to interface directly with a variety of mass spectrometry (MS) systems
- Ease of automation using standard technologies

Because of its ability to separate and identify molecules with much lower molecular weights, HPLC is the technology of choice for plant protein identification through peptide analysis, often after a 2DE run isolates the particular protein for tryptic digestion. Where HPLC systems have traditionally lagged, however, is in this initial separation of proteins from complex vegetable mixtures, especially because they lack the visualization step that a gel display provides.

HPLC systems, however, are catching up to 2DE even in the area of complex protein mixtures. Multidimensional HPLC for protein analysis primarily relies on tandem or multiple liquid chromatography (LC) combined with tandem MS. Given the sensitivity of the initial LC steps and the fact that everything is done in liquid in-line, such systems are not only easily automated but also capable of separating even the low-abundance and membrane proteins. These proteins cause difficulties in 2DE analysis because of poor yields, difficult visualization, or interference with the gel runs. And, with new informatics tools, analysis of the chromatography data can be presented in more user-friendly displays of multidimensional runs [1,2].

18.2 AMINO ACID ANALYSIS OF VEGETABLE EXTRACTS

18.2.1 Main Goals

Amino acids are considered nitrogen donor ligands in the phenomenon of hyperaccumulation of heavy metals in plants [3]. The involvement of heterocyclic nitrogen donors (amino acids) in the uptake of Ni^{2+} by plants has also been suggested [4]. Most studies failed to provide causative relationships between amino acid levels and metal hyperaccumulation (or tolerance). The production of particular amino acid(s) and their concentrations in hyperaccumulating plants, as a consequence of exposure to elevated levels of heavy metals (or hyperaccumulation), appears to be a species-specific phenomenon and is not necessarily present in all the metal-hyperaccumulating plants. For example, an increase has been noted in the concentration of histidine in the xylem sap of three Ni hyperaccumulators, *Alyssum lesbiacum*, *A. murale*, and *A. bertolonii*, in proportion to external Ni application [5]. Similarly, nickel application in the hydroponic solution elicited a large and proportional increase in free histidine in the leaves of *Berkheya coddii* [6]. In contrast, in bulk leaf extracts of four Ni hyperaccumulators, only a slight change has been evidenced in the total amino acids over a wide range of leaf Ni concentrations [7]. Any relationship between external Ni levels and amino acid content has been found in the xylem fluids (or above-ground tissue) of hyperaccumulating plants, so that the relationship between amino acid content and metal accumulation, if any, is

obscure and enigmatic [7,8]. Furthermore, while commenting on the need to minimize the risk of artefactual observations following tissue processing, Homer et al. emphasized the need to examine specific samples such as xylem sap for amino acid determinations, particularly for Ni hyperaccumulators [7].

A total of 19 free amino acids were detected in the xylem sap of field-collected, young plants of *Stackhousia tryonii* Bailey, a rare herbaceous Ni hyperaccumulator endemic to the serpentine soils of central Queensland (Australia), grown in a glasshouse for 20 weeks [9]. It has been observed that in the xylem sap of low-Ni-treated plants, glutamine was the dominant amino acid, with concentrations reaching up to 11 mM and accounting for half of the total amino acid concentrations in the xylem sap. Glutamic acid, alanine, and aspartic acid were also present, but their concentrations were only 2.8, 1.2, and 1.1 mM, respectively. All other amino acids were present in very low concentrations (<1.0 mM). In the xylem sap of high-Ni-treated plants, glutamine, glutamic acid, alanine, and aspartic acid were the major amino acids at 4.1, 3.4, 2.9, and 1.5 mM, respectively. Glutamine accounted for nearly one-quarter of the total amino acid concentration in the xylem sap. The individual concentrations of all other amino acids were below 1.0 mM. The total concentration of free amino acids in the xylem sap was similar in low- (21.7±3.7 mM) and high-Ni-treated plants (17.9±5 mM).

Amino acid profiles differ among plant families and genera, and their total concentration is related to maturity and nitrogen (N) nutrition of the plant [10]. Some authors found large genotypic differences in amino acid concentration among tall fescue genotypes, but in neither of the preceding studies were amino acid profiles corrected for differences in N concentration [11]. Relative proportions of each amino acid are often similar within a species or genus when corrected for plant N, unless there are large differences in total N or in nonprotein N in the tissue. In a review of postingestive feedback [12], it has been noted that deficits or imbalances of amino acids decrease intake and cause feed aversions in lambs (*Ovis aries*). Thus, forages containing higher concentrations of malate and citrate or perhaps certain amino acids might enhance flavor and nutrient utilization and provide positive postingestive feedback affecting ruminant preference. Malate, citrate, and amino acid concentrations have been determined in endophyte-free tall fescue (*Festuca arundinacea* Schreb) and, those concentrations have been related to cultivar, harvest time, and grazing-animal preference [13]. "Barcel," "Kenhy," "Kentucky-31," "Missouri-96," "Mozark," and "Stargrazer," and the two accessions C1 and HiMag, were established in three replicates within each of three pastures. The results of this research support the hypothesis that concentrations of amino acids in plants vary with total protein in the tissue, and this is reflected by changes in the concentration of total N (except perhaps if nonprotein N is high; e.g., high nitrate). It follows that any statistical analyses of data on amino acid concentration should be corrected for N concentration as a covariate.

In broccoli (*Brassica oleracea* L. var. *italica* Plenck) at harvest, catabolic senescence leads to degradation of proteins with liberation and accumulation of amino acids [14,15]. The changes in concentrations of non-protein-bound amino acids seem to be due to increased proteolysis and amino acid interconversion [14]. Recently, it was reported that total free amino acid content increased twofold when broccoli plantlets were stored for 8 days in anaerobic or aerobic conditions at 20°C. However, individual amino acids were not measured [16]. Other authors reported that treatment of lettuce with 15% or 20% CO_2 in air affected the concentration of γ-aminobutyrate and glutamate but not the other nine amino acids they measured in their study [17].

Potato tubers from pots to which differing amounts of nitrogen had been added were analyzed [18]. Increasing levels of nitrogen fertilizer, applied in inorganic or organic form, increased the concentrations of asparagine and glutamine in the tuber dry matter as well as their amounts per pot. Amide concentrations are significantly correlated to those of crude protein ($P < -0.001$) and nitrate ($P < -0.01$).

Moreover, amino acids have been identified as the precursors of a class of secondary plant metabolites, the glucosinolates, but there is no report on the relationship between the two in vivo. In an attempt to evidence the relationship between free amino acids and glucosinolates, the behavior of these compounds was investigated in 11 broccoli cultivars grown in early (April–July) and

late (August–January) seasons [19]. Free amino acids and glucosinolates were quantified by HPLC in primary and secondary inflorescences at maturity stage. Analysis of the data revealed that no significant correlation exists between free amino acids and glucosinolates, suggesting that the precursors of these compounds might be used during the first step of the biosynthetic process to form intermediates, the aldoximes and thiohydroximates, which will then be used in the second stage of the biosynthesis to form, apart from glucosinolates, other secondary plant metabolites.

Gardener and Gillman [20] observed that in several species of flowering plants, the total concentration of amino acids varied greatly (average coefficient of variation 0.65), but composition was much less variable (average correlation among samples from a single species 0.85). The absolute concentration of individual amino acids turned out to be much more variable than the relative abundance (coefficients of variation 0.98 and 0.77, respectively; $N = 544$, $t = 16.98$, $P < 0.001$). When amino acids that occurred in only low relative abundance (<1%) were removed from the analysis, the difference was even more marked (0.78 and 0.51, respectively; $N = 344$, $t = 15.13$, $P < 0.001$). The results highlighted the need for large sample sizes when making observations concerning the absolute amounts of amino acids in nectar and for sensitive analyses of the composition, as even small changes might be biologically significant.

Amino acids were also determined in nutritional serum, tobacco extract, and wine with a single calibration curve [21]. The chemiluminescent nitrogen detector (CLND) was useful for determining amino acid stoichiometry of peptide hydrolysates; no calibration curves were needed if the molecular weight of the peptide was known. Detection limits varied from 0.0025 to 0.0075 mM (0.33–0.86 mg/L) depending on the amino acid and its retention time.

18.2.2 Methodologies

There are many ways to identify and quantify amino acids in sample extracts. A quick and simple estimate of total amino acid concentration is based on the reaction of reduced ninhydrin with the amino group [22]. A more accurate technique uses precolumn derivatization and HPLC to facilitate the identification of individual amino acids [23–25]. The derivatized samples can be separated and quantified by fluorescence [25] or UV detection [24]. The details of the HPLC protocol used depend on the type and availability of HPLC systems. It should also be noted that polypeptides and proteins can be hydrolyzed into their individual amino acids by boiling them in 6 N HCl under vacuum for 20–24 h [23]. Such a treatment results in the conversion of tryptophan, glutamine, and asparagine into their respective acids, and these amino acids are therefore not measured. However, the hydrolysis step is often not included in the protocols, when the intention is to determine the identity and quantity of free amino acids (amino acids present in peptides or proteins are labeled *bound amino acids*). An alternative protocol includes a step that removes polypeptides and proteins to prevent contamination of the HPLC column or coelution [25].

Both primary and secondary amino acids were analyzed in one run. The amino acid composition of proteins can be used to detect adulteration of foodstuffs. Detection of potentially toxic amino acids is also possible through such analysis. Through the use of chiral stationary phases as column material, D- and L- forms of amino acids can be separated and quantified. HPLC in combination with automated on-line derivatization is now a well-accepted method for detecting amino acids owing to its short analysis time and relatively simple sample preparation. Hydrolyzation with HCl or enzymatic hydrolysis is used to break protein bonds. For example, an HPLC method was used in the analysis of secondary and primary amino acids in beer with precolumn derivatization and fluorescence detection [26].

Free amino acids in the xylem sap sample from young plants of *Stackhousia tryonii* were analyzed using a Waters AccQTag amino acid analysis system [9]. The amino acids in the xylem sap were separated by a sensitive precolumn derivatization technique that is used with reversed-phase HPLC (RP-HPLC) and quantified by fluorescence or UV detection. Before separation, amino acids were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) [27]; they were

then transferred to the HPLC autosampler for analysis. The HPLC consisted of (in series): a separation module (Waters Corporation, MA, USA), a fluorescence detector, and a dual λ absorbance detector. The control and analysis software was Waters Empower Pro. For all separations, a Waters AccQTag column (15 cm × 3.9 mm ID) was used. The column and autosampler temperatures were maintained at 37°C and 10°C, respectively. The sample injection volume and flow rate were 5 μL and 1 mL/min, respectively. The fluorescence detector wavelengths were 250 nm (excitation) and 395 nm (emission). The wavelength of the UV/VIS (ultraviolet/visible) detector was set at 254 nm.

Amino acid concentrations were determined on plant tissue harvested in May and September 1993 from three replicates of Barcel, HiMag, Kenhy, and Mozark cultivars of *Festuca arundinacea* [13]. Protein in 30–50 mg of freeze-dried plant material was hydrolyzed according to AOAC methods 982.30, 988.15, and 985.28 [28]. Individual amino acids were determined by postcolumn detection of their ninhydrin derivatives (570 and 440 nm) after separation by ion-exchange HPLC (IE-HPLC). Nor-leucine and taurine were added before the hydrolysis to assess stability and recovery of acid-stable and sulphur amino acids (cysteine and methionine), respectively. Recoveries were typically better than 95% across the range of amino acids. Instrumentation consisted of a Shimadzu LC 10A autosampler and an HPLC system coupled with a Pickering Laboratories PCX3100 derivative system (Pickering Laboratories, Mountain View, CA, USA) equipped with a Pickering 3 × 250 mm, 8-mm sodium cation-exchange column. Operating conditions were optimized to allow separation and quantification of 18 amino acids in plant protein. Separate programs were used to analyze acid-stable amino acids, sulphur amino acids, and tryptophan. Amino acid concentrations in the hydrolysate were determined relative to the response factors of amino acid standards (Pierce amino acid calibration mixture H; Perstorp, Inc., Florence, MA, USA). Using ninhydrin detection and measuring absorbance changes at 570 nm, sensitivity was 0.2 pmol (3 s detection limit). Repeated amino acid analyses of a standard casein sample showed that precision was better than 3% at 5 nmol. The coefficient of variation of repeated analyses of amino acids in standard casein ranged from 4.5 to 6.0%, with the exception of arginine, histidine, and valine (which ranged from 6.0 to 7.5%); isoleucine, methionine, and tyrosine (which ranged from 7.5 to 9.0%); and cyst(e)ine (19%).

Other authors determined the levels of amino acids extracted from freeze-dried, finely ground broccoli powder separated on semi-micro columns followed by *o*-phthaldialdehyde (OPA) derivatization and HPLC separation [29]. Individual amino acids were identified by comparing the HPLC retention times of the unknown sample with those of individual standard compounds. The internal standards, *p*-hydrazine and nor-valine, were used for calculations of basic and neutral/acidic amino acid concentrations, respectively [30]. Hippe reported the rapid determination by HPLC of free asparagine and glutamine in potato tubers [18].

An LC procedure has been developed for the separation and quantification of amino acids in some vegetables by using methyl benzoate as the internal standard [31]. The dinitrophenyl (DNP)-amino acid derivatives were separated on a reversed-phase column (LiChrosorb RP-18, 5 μm), eluted with a linear gradient of 20–75% (v/v) acetonitrile in 1% (v/v) glacial acetic acid in water. The column effluent was monitored at 254 nm. A total analysis of DNP-amino acids was completed in less than 55 min. For 14 amino acids and asparagine plus glutamine, separation and quantification were satisfactory. This technique has been used effectively to analyze amino acids in biological samples by the internal standard method.

In *Fragaria* and *Citrus*, Ramamurthy and Luedders carried out the separation of glutamic acid, glutamine, aspartic acid, and asparagine on a reversed-phase column in an HPLC method [32]. The conditions for the separation were optimized. Two columns with chain lengths C-8 and C-18 were tried. The components of the mobile phase were chosen so as to obtain maximum retention of the extremely hydrophilic amino acids to be separated. The final separation was done on a RP-C-18 column using 25 mM phosphate (pH 2.1) containing 5 mM heptyl sulfate and 0.25% methanol. The amino acids were detected, nonderivatized, by monitoring the column effluent at 200 nm.

Moreover, the free amino acids in *Zea mays* roots were separated by using an RP-HPLC procedure developed for rapid separation and quantization of free amino acids as *o*-phthaldialdehyde

derivatives [33]. A two-step isocratic solvent system was used that enabled an accurate analysis at nanomole level. However, two major disadvantages of this procedure were the lack of proline reaction and the coelutions of threonine/glycine and tryptophan/methionine.

Major amino acids in xylem exudates of tomato plants were separated by RP-HPLC and quantified by UV detection [34]. Before separation, amino acids were converted into their phenylisothiocyanate (PITC) derivatives. In a single run, aspartic acid, glutamic acid, serine, glutamine, histidine, threonine, alanine, tyrosine, valine, methionine, cysteine, isoleucine, leucine, phenylalanine, and lysine were separated and detected down to the pmol level. Unresolved peaks were obtained for asparagines and glycine and for arginine and proline.

Thirty species of flowering plants were analyzed for floral nectar amino acid composition [20]. HPLC was used in conjunction with AccQtag derivatization to produce accurate and precise data. Recently, underivatized amino acid analysis was demonstrated with ion-pair RP-HPLC coupled with a CLND [21]. Volatile ion-pairing reagents (perfluorinated carboxylic acids) were shown to be compatible with this mode of detection. The linearity and equimolarity of this mass-dependent detector have been confirmed.

18.3 PEPTIDE ANALYSIS OF VEGETABLE EXTRACTS

18.3.1 Main Goals

Plant peptides perform a variety of functions. Up to now, genetic as well as biochemical studies have demonstrated that (1) extracellular peptides have the function of signaling between plant cells and organs and defending against pathogen attack; and (2) intracellular ones often show physiological functions or may merely be the products of general proteolysis [35].

A substantial proportion of the plant peptides are secretory and act as local signals mediating cell-to-cell communication. Specific receptors for several peptides were identified as being membrane-localized receptor kinases, the largest family of receptor-like molecules in plants. These findings illustrate the importance of peptide signaling in the regulation of plant growth, functions that were previously ascribed to the combined action of small lipophilic compounds referred to as "traditional plant hormones" [36].

Long-distance signaling between roots and shoots and the induction of appropriate responses is essential for the optimal functioning of higher plants in their continually changing soil and aerial environments. Most of the available evidence concerns chemical signaling via the traditional plant hormones. For example, leaf epinastic responses to root flooding have been related to increased root-to-shoot xylem transport of the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) [37,38]. Bioactive peptides are now considered major components of signaling for defense, leaf shape, self-incompatibility, nodule development, organ abscission, cell division, cell differentiation, and root growth; some evidence supports the occurrence of long-distance signaling via phloem transport of small RNA and signal peptides such as systemins [39–41].

Systemins are 18 amino acid peptides produced in plants responding to the stress of localized wounding; they induce a systemic activation of plant defense proteins such as the serine protease inhibitors, which may upset protein digestion in predator insects. However, the possibility that systemin itself (or other peptides) undergoes long-distance phloem transport throughout the plant remains controversial [42]. The xylem sap of monocot and dicot species is known to readily transport water, mineral ions, amino acids, carboxylic acids, protons, and traditional nonpeptide hormones from the roots to the shoots [43,44]. It also transports a range of large enzyme proteins including serine, aspartyl, and cysteine proteases [45–50]. Tomato systemin is the first identified plant-signaling peptide. It is composed of 18 amino acids and is able to induce a systemic wounding response in tomato plants attacked by herbivores. Although more than 100 diverse plant species respond systemically to herbivore attacks [51], homologues of systemin could be identified only in some other Solanaceae [52] but, strikingly, not in tobacco, which also belongs to the Solanaceae.

It was known that tomato systemin could activate a mitogen-activated protein (MAP) kinase in suspension culture cells and induce an alkalinization of the medium [53]. In tomato plants, systemin is able to induce expression of proteinase-inhibitor protein-encoding genes. These properties of tomato systemin have been exploited to search for a peptide with systemin-like properties in extracts of herbivore-attacked tobacco leaves using a tobacco suspension culture [54].

A group of peptides, phytochelatins (PCs), plays an essential role in heavy-metal detoxification in plants. PCs chelate heavy metals, and then PC-metal complexes are translocated across the tonoplast and sequestered in vacuoles. PCs are synthesized from glutathione by the enzyme PC synthase (PCS).They are activated post-translationally in the presence of metal ions. Heavy metals do not bind directly to the enzyme to activate PC biosynthesis but instead act as substrate ligands for a bisubstrate-substituted enzyme transpeptidation reaction in which free glutathione and its corresponding heavy-metal thiolate are cosubstrates [55–57]. A study aimed at establishing a method for purification and performing enzymatic characterization of the PCS from *Arabidopsis thaliana* was carried out [58]. Moreover, the PCS gene was expressed in *E. coli* and *S. cerevisiae* to enhance tolerance to toxicity from cadmium ion and mercury. Because signal transduction cascades are hypothesized to have recruited metal homeostasis factors, a better understanding of plant metal handling might also lead to new insights into other fundamental aspects of life physiology. In addition to the main function of heavy-metal detoxification, PCs may have a function as an antioxidant.

Finally, there is a family of peptides named plant lipid transfer proteins (LTP). They are cationic peptides, subdivided into two families, which present molecular masses of around 7 and 10 kDa. These peptides were thus denominated due to their ability to reversibly bind and transport hydrophobic molecules in vitro. Both families possess conserved patterns of eight cysteine residues, and the three-dimensional structure reveals an internal hydrophobic cavity that comprises the lipid binding site. Based on the growing knowledge regarding structure, gene expression and regulation, and in vitro activity, LTPs are likely to play a not-well-known role in key processes of plant physiology [59].

18.3.2 Methodologies

RP-HPLC has become the method of choice for the purification of peptides and small proteins ($M_r < 10{,}000$ Da) from vegetable sources. The technique combines high resolution and recovery with ease and speed of operation and is applicable to a wide range of peptides with different physicochemical properties.

Bioactive signal peptides of plant and microorganism origin have been shown to block a proton pumping ATPase, thereby reducing the outward flux of protons to the apoplast and causing an alkalinization of the medium of suspension-cultured cells [60,61]. The regulation of root and shoot growth has also been partially associated with altered transport of protons into (expanding) cell walls [62–67]. A sensitive bioassay based on induced alkalinization of the medium of suspension-cultured wild tomato cells has been developed and used conveniently to detect nanomolar concentrations of several different bioactive signal peptides in purified extracts from plant shoots [68–71]. Neumann standardized a cell-suspension alkalinizing assay to search for the presence of signal peptide activity in xylem sap naturally exuded from the roots of detopped greenhouse-grown tomato plants under well-watered or saline conditions [72]. Batches of 6–10-week-old plants were used for exudate collection. Some plants in each batch were salinized by irrigating with nutrient solution containing 100 mM NaCl for 2 days. At 30 minutes before sap collection, the salinized soil was thoroughly flushed with nutrient solution containing 20 mM NaCl in order to decrease osmotic restraints to root pressure development. The shoots of control or salinized tomato plants were excised with a fresh razor blade 2–3 cm above the soil level and discarded. The cut stem surfaces protruding from the soil were repeatedly rinsed with streams of water and blotted before collection of the exuding xylem sap droplets by use of a pipette. Sap was stored on ice in graduated plastic tubes, and a pooled volume of 1.5 mL or more was collected after about 2 h from batches of 5–20 plants per treatment. Protein levels in the sap of control plants ranged between 70 $\mu g \bullet mL^{-1}$ and

100 μg•mL^{-1}. Collected sap was desalted and partially purified by passage through C18 Sep-Pak cartridges (Waters, Milford, MA, USA) and elution of the adsorbed activity with 1 mL aliquots of 25%, 50%, and 75% acetonitrile in 0.1% trifluoroacetic acid (TFA). The pooled eluates were freeze-dried and suspended in 0.1% TFA prior to assay of alkalinizing activity using *L. peruvianum* cell suspensions. In a preliminary attempt [72], C18 column elution profiles and UV absorption were used to identify signal peptides in extracts from plant leaves. A root xylem sap preparation was therefore analyzed using a narrow-bore HPLC C18 column (2.1 mm ID, Vydac 218TP52, Hesperia, CA, USA) equilibrated with 0.1% TFA in water at a flow rate of 0.25 mL/min and a gradient to 50% acetonitrile in 0.1% TFA. Fractions eluting from the C18 column at 1-min intervals between 20 min and 70 min exhibited various UV-absorbing components when measured at 214 nm. So the alkalinizing activity in a xylem sap preparation was found to elute during a C18 column chromatography run at concentrations of nonpolar solvent previously shown to elute bioactive plant peptides, and the activity was associated with UV absorption at 214 nm (indicative of peptide carbonyl linkages).

A method for the analysis of cyclic peptides, like Pseudostellarin, extracted from the traditional herbal Chinese medicine, *Pseudostellaria heterophylla* (Miq.) Pax, was established by HPLC–atmospheric pressure chemical ionization (APCI)–MS. Pseudostellarins have shown potent tyrosinase- and melanin-production inhibitory activities. Most of the work already reported in the literature has focused on the extraction, separation, and identification of the structures by nuclear magnetic resonance (NMR) analysis and off-line MS. A total sample of 0.5 g of the fine-ground powder was accurately weighed and extracted with 50 mL of methanol in an ultrasonic bath for 30 min and filtered. This extraction was repeated twice. The combined filtrate was evaporated to dryness in vacuo. The residue was then dissolved in methanol and diluted to 10 mL in a volumetric flask and filtered through a 0.45-μm filter membrane before analysis. Aliquots (20 μL) were automatically injected into the HPLC system. A standard stock solution of 1 mg•mL^{-1} of Pseudostellarin was prepared by dissolving accurate amounts of pure standard in methanol. A working solution of the standard was achieved by several dilutions in water (HPLC grade). The stock and working standard were stored at 4°C. This solution was used for preparing the calibration curve and for the determination of Pseudostellarin in real samples. The separation of Pseudostellarin was carried out using a HPLC system, consisting of a vacuum degasser, autosampler, and binary pump (Agilent Series 1100, Agilent Technologies, USA), equipped with a reversed-phase C18 analytical column (Eclipse XDB-C18, 4.6 mm × 50 mm, 5 μm, Agilent). The mobile phase consisted of pure water–acetonitrile. The percentage of acetonitrile in the mobile phase was programmed as follows: 10% (0 min)–20% (10 min)–30% (40 min)–45% (55 min), and the flow rate was 0.4 mL/min. Column temperature was maintained at 30°C. The injected sample volume was 20 μL. A 10-min postrun time back to the initial mobile-phase composition was used after each analysis [73].

Matsumoto and colleagues assayed PCS activity in *A. thaliana* extracts [58] according to the method reported by other authors [74]: in reaction media containing crude enzyme fraction (50 mg protein), 0–20 mM GSH (reduced glutathione), 10 mM 2-mercaptoethanol, 200 mM Tris–HCl buffer (pH 8.0), and 0–0.75 mM $CdCl_2$ at 37°C for 30–180 min. The extracted sample was neutralized by the addition of 3.6 N HCl to stop the reaction. The postcolumn derivatization method involving DTNB (5,5'-dithiobis-2-nitrobenzoic acid) was used for sensitive detection of PCs. The clear supernatant was subjected to RP-HPLC analysis (column: Waters Symmetry 300 C18, 5 μm, 4.6 × 150 mm; solvent system: A: 0.02% TFA, 5.0 mM octanesulfonic acid, B: 30% acetonitrile, 0.02% TFA; gradient 13–100% B in 30 min; flow rate: 1.0 mL/min), and the eluted sample was continuously mixed with the thiol-reactive solution, containing 10% acetonitrile, 75 mM DTNB, and 100 mM potassium phosphate buffer (pH 8.0), to detect the PC and GSH at 412 nm using Jasco HPLC UV-2075 Plus.

In another study, a method was proposed for the detection and quantification of unbound phytochelatin in plant extracts of *Brassica napus* growth with different levels of mercury via HPLC coupled to electrospray tandem MS and inductively coupled plasma MS in parallel [75]. The roots

and shoots of rape plants were analyzed separately for Hg accumulation. Metal accumulation in rape plants was observed mainly in the roots. The method developed was further applied to the characterization and determination of PCs in extracts of rape, a plant species reported to biosynthesize PCs when exposed to different levels (0–1000 mM) of Hg^{2+}. The plant extracts were analyzed directly by HPLC-ESI-MS/MS. Rape (*Brassica napus*) plants were grown under semihydroponic conditions, using half-strength Hoagland nutrient solution, with sand as a substrate. Three pots per treatment with three replicates (plants) per pot were used. Seeds germinated in sand. Seedlings were grown for 6 weeks. The Hg concentrations applied were 0, 50, 125, 250, 375, 750, and 1000 mM $HgCl_2$. The first approach to the characterization of PCs by LC-MS was done in a single-quadrupole mass spectrometer (Platform). Although all the studied PCs emitted a signal in full-scan mode, not enough sensitivity was attained for the sample even in single-ion-monitoring (SIM) mode. Therefore, the study was finally performed using the triple-quadrupole mass spectrometer API 3000 (Applied Biosystems PE Sciex). This instrument allows a more accurate characterization through product-ion-scan experiments and a more sensitive quantification through multiple reaction monitoring (MRM) experiments.

Different concentrations (2, 2.5, 3, and 3.5 mM) of synthetic PC_2, PC_3, and PC_4 were used during preliminary assays for further detection of PCs in experimental samples. A chromatogram was obtained for each PC and for the mixture of synthetic PCs ($PC_2 + PC_3 + PC_4$) as described in reference [76]. Quantitation was performed using the time-scheduled SIM mode with a dwell time of 100 ms. A Luna C18 Phenomenex column (250×4.00 mm, ID 5 mm) equipped with a Securityguard C18 Phenomenex (4×3 mm ID) was used. Gradient elution was done with water with 0.1% acetic acid (solvent A) and acetonitrile with 20% solvent A (solvent B). Separation was achieved with a linear gradient of 2% solvent A to 100% solvent B at a flow rate of 1.0 mL/min, and a column temperature of 30°C.

Efforts to replace costly, undefined serum products in cell culture media have led to the study of low-cost protein hydrolysates from animal tissues, milk products, microorganisms, and plant tissues [77]. Use of animal-origin serum-replacement products has been reevaluated due to increased concern about the potential for contamination from adventitious agents. It was reported that protein hydrolysates can either partially or fully replace serum as a concentrated balanced nutrient mixture [78]. The cell culture media developed by Sigma-Aldrich are frequently used in large-scale protein production by the biotechnology industry, which increasingly requires serum-free media. In an effort to find a serum substitute for cell culture media, wheat gluten hydrolysates have been evaluated [79]. Wheat gluten hydrolysates are a known source of high levels of small peptides and larger oligopeptides and are particularly rich in stable glutamine [80]. In such studies, a positive effect of wheat gluten hydrolysates on cell culture productivity was observed, but the degree of productivity enhancement varied between batches of wheat gluten hydrolysate. In general, the chemical composition of protein hydrolysates was somewhat ill-defined. In order to control the quality of cell culture media formulated with protein hydrolysates, one must be able to control the quality of the hydrolysates. The use of peptide mapping by RP-HPLC was reported in order to screen wheat gluten hydrolysates as potential raw materials in serum-free cell culture media [79].

An RP-HPLC method with photodiode array detection has been developed for peptide mapping of wheat gluten hydrolysate to examine lot-to-lot variability. Significant differences noted in the profile were correlated to observed differences in the biological activity of cells grown in medium containing different lots of hydrolysate. The profiling method can be used to monitor changes in hydrolysate peptide composition due to variations in processing conditions. The HPLC system consisted of a Waters 2690 Alliance separation module equipped with a column heater coupled to a Waters 996-photodiode array detector. A Supelcosil C18 column (250×4.6 mm; 5 mm) was used for the separation of the hydrolysates. Data collection and processing were performed using Waters Millennium software 3.05.01. Mobile phase A was prepared by adding 1.0 mL TFA–1000 mL HPLC-grade water; mobile phase B was prepared by adding 1.0 mL TFA–1000 mL acetonitrile. Separation of hydrolysate components was performed using a 90-min linear gradient from 0% to

30% B. The column was washed for 5 min with 100% B and reequilibrated for 15 min between injections. The flow rate was 0.7 mL/min, and the column temperature was maintained at 25°C. UV data were recorded from 210 to 285 nm. The injection volume was 100 µL. The wheat gluten hydrolysate was prepared at 5 g/L in hot (56–60°C) HPLC-grade water, filtered through a 0.2-mm filter, and diluted to 2.5 g/L with mobile phase A for injection.

This HPLC method was developed to qualitatively evaluate differences in the peptide composition of wheat gluten hydrolysates. The instrument method employed a shallow gradient because of the high concentration of small peptides in the hydrolysates. Although it is not conventional to initiate a gradient separation with a 100% aqueous mobile phase, it was necessary to achieve retention of several hydrophilic components. Initially, the absorbance at 214 nm was monitored because it is generally accepted as the standard peptide-mapping wavelength. Lots of wheat gluten hydrolysate that resulted in different cell culture productivity enhancements showed different elution profiles at 214 nm. It was evident that such hydrolysates contained a considerable number of distinct peptide components. The nearly identical overlay of the lots of hydrolysate at 214 nm did not provide any significant differentiation between the samples. Because the spectral range from 210 to 285 nm was collected throughout the separation using a photodiode array detector, chromatographic profiles at wavelengths other than 214 nm provided further differences between the hydrolysate samples. The UV spectra of proteins and peptides were greatly dependent on the spectra of the amino acids of which they were composed [81]. The absorbance at 280 nm is often used for protein and peptide detection based on the aromatic amino acid residues.

18.4 PROTEIN ANALYSIS OF VEGETABLE EXTRACTS

18.4.1 Main Goals

While proteomics research is advanced in animals and yeast, plant proteomics is still at the initial phase [82–84]. The progress in plant proteomics has largely been made by analyzing various functional aspects of proteins such as posttranslational modifications, protein–protein interactions, activities, and structures. General HPLC methodologies have been adopted by several authors in order to improve the knowledge of plant proteomics. Since the resolution of protein peaks in an elution profile is limited by factors such as abundance, size, and other chromatographic properties, the complete proteome has been fractionated into subproteomes such as subcellular compartments, organelles, and multiprotein complexes to improve sensitivity and resolution and to reduce the overall complexity.

High-throughput separations are an essential prerequisite for the detection and analysis of proteins by HPLC. Together, efficient separation and MS can lead to the identification of thousands of proteins in a vegetable sample, cell, or tissue and help build proteome maps that can be used to define a cell type or cellular state. Although 2D gels have been successfully used to separate proteins for subsequent MS analysis, alternative separation efficiencies and, consequently deeper results could be obtained with HPLC or other separation techniques that improve throughput. HPLC analysis is aimed toward plant scientists who have special separation needs due to the nature of plant cells and who could benefit from knowing options and requirements for adopting alternative separation protocols. Through the various sample-processing and protein-separation strategies, plant biologists should be able to improve the quality of their proteomic reference maps and gain new information about the proteins that define plant cells.

The field of proteomics with new analytical approaches such as two-dimensional LC hyphenated to MS opens novel opportunities in plant protein knowledge such as elucidation of biosynthetic pathways or clarification of plant response to stress, as, for example, the production of heat shock proteins [83].

The development of nondenaturating HPLC in the field of genomics in 1993 was revolutionary [85–88]. The core of this HPLC method consists of a nonporous polystyrene-divinylbenzene (PS-DVB) stationary phase, which allows the fast separation and safe determination of DNA

mutations and is therefore used in clinical centers worldwide for the analysis of breast cancer [87]. This new technique not only allows the determination of mutations such as mamma carcinoma but can also be used in the field of plant analysis, especially in the field of cell culture and plant breeding systems. The combination of the three big domains, that is, metabolomics, proteomics, and genomics, opens new opportunities such as the study of the plant answer to stress factors from outside, for example, the analysis of genetic regulations, protein and enzyme synthesis, and finally the production of specific ingredients.

Some examples of protein analysis through different HPLC methodologies are reported in the following.

18.4.2 Methodologies

Hydrolyzed plant proteins are widely used as ingredients in culinary products for their glutamate-like ("umami") taste. Schlichtherle-Cerny and Amado prepared three hydrolysates from wheat gluten using different enzymatic approaches [89]. Comparison of their taste profiles revealed the enzymatic hydrolysate of an acid-deamidated wheat gluten (WGH-3) to be the least bitter of all and to elicit an intense glutamate-like taste. Its umami taste intensity was similar to that of an enzymatic hydrolysate in which glutaminase had been employed to convert free glutamine to glutamic acid and which had a threefold higher concentration of free glutamate. Reconstitution studies based on the results of the chemical analysis of WGH-3 and sensory comparison of the model solution and WGH-3 indicated that other components in addition to glutamate and organic acids contribute to its glutamate-like taste. WGH-3 was fractionated by gel permeation chromatography and RP-HPLC, and two fractions with a pronounced glutamate-like taste were obtained. In one of them four pyroglutamyl peptides were tentatively identified as pGlu-Pro-Ser, pGlu-Pro, pGlu-Pro-Glu, and pGlu-Pro-Gln. Apparently, these peptides were formed by cyclization of the N-terminal glutamine residues during the preparation of the hydrolysates.

Chromatographic fractionation of crude extracts (C8 extracts) from the protein-enriched flour of commercial field peas (*Pisum sativum* L.) has been shown to yield peptide mixtures related to the pea albumin 1b (PA1b) family of cysteine-rich plant peptides [90]. The mixtures were obtained initially by flash chromatography with silica gel. Following elution of soyasaponins and lysolecithins, the end fractions obtained with the use of two flash chromatographic solvent systems displayed activity in a flour-disk antifeedant bioassay with the rice weevil (*Sitophilus oryzae* L.). Chemical properties of these mixtures were compared by thin-layer chromatography, HPLC, IR, MS, and amino acid analyses. The low-molecular-weight proteins of C8 extracts, with average masses of 3752, 3757, and 3805 Da, were isolated by anion-exchange chromatography. Samples enriched in the protein of mass 3752 were isolated by cation-exchange chromatography. Reduction plus alkylation experiments in combination with electrospray ionization MS showed that C8 extracts contained about 10 small proteins, and, like PA1b, each of them possessed six cysteine residues (three disulfide bonds). Disulfide bond reduction with 2-mercaptoethanol destroyed the antifeedant activity. The native proteins of C8 extracts were found to be resolved into nine peaks with XTerra HPLC columns operating at alkaline pH. These columns were employed to assess the distribution of pea proteins in the isolated fractions, with photodiode array and electrospray detection.

A range of studies have compared the level of nutritionally relevant compounds in crops from organic and nonorganic farming systems, but there is very limited information on the effect of farming systems and their key components on the protein composition of plants. An attempt to address this gap was made by quantifying the effects of different farming systems and key components of such systems on the protein profiles of potato tubers [91]. Tuber samples were produced in the Nafferton factorial systems study, a group of long-term, replicated factorial field experiments designed to identify and quantify the effect of fertility-management methods, crop-protection practices, and rotational designs used in organic, low-input, and conventional production systems. Protein profiles were determined by 2DE and subsequent protein identification by HPLC-ESI-MS/MS. Principal-

component analysis of 2DE data showed that only fertility-management practices (organic matter vs. mineral fertilizer based) had a significant effect on protein composition. Quantitative differences were detected in 160 of the 1100 tuber proteins separated by 2DE. Proteins identified by MS are involved in protein synthesis and turnover, carbon and energy metabolism, and defense responses, suggesting that organic fertilization leads to an increased stress response in potato tubers.

Adhesion of pollen grains to the stigmatic surface is a critical step during sexual reproduction in plants. In *Brassica*, S locus-related glycoprotein 1 (SLR1), a stigma-specific protein belonging to the S gene family of proteins, has been shown to be involved in this step. However, the identity of the interacting counterpart in pollen and the molecular mechanism of this interaction have not been determined. Using an optical biosensor immobilized with S gene family proteins, strong SLR1-binding activity was detected in pollen coat extracts of *Brassica campestris* [92]. Two SLR1-binding proteins, named SLR1-BP1 and SLR1-BP2, were identified and purified by the combination of SLR1 affinity column chromatography and RP-HPLC. Sequence analyses revealed that these two proteins (i) differ only in that a proline residue near the N terminus is hydroxylated in SLR1-BP1 but not in SLR1-BP2, and (ii) are members of the class A pollen coat protein (PCP) family, which includes PCP-A1, an SLG (S locus glycoprotein)-binding protein isolated from *Brassica oleracea*. Kinetic analysis showed that SLR1-BP1 and SLR1-BP2 specifically bound SLR1 with high affinity (K(d) = 5.6 and 4.4 nM, respectively). The SLR1-BP gene was specifically expressed in pollen at late stages of development, and its sequence is highly conserved in *Brassica* species with the A genome.

Different types of antimicrobial peptides were identified in seeds from different plant species [93]. The aim of this study was to isolate and characterize small proteins present in chili pepper seeds (*Capsicum annuum* L.) and evaluate their toxic activity against some yeast species. Initially, proteins from seed flour were extracted in phosphate buffer, pH 5.4, for 3 h at 4°C, and the pellet obtained at 90% saturation with ammonium sulfate was heated at 80°C for 15 min. The resulting suspension was clarified by centrifugation, and the supernatant was extensively dialyzed against water; the peptide-rich extract was then named F/0-90. Cation-exchange HPLC was performed to separate low-molecular-mass proteins. One of the resulting fractions, named F3, enriched with basic proteins of 6–16 kDa, was submitted to reversed-phase chromatography in a C2/C18 column by HPLC, resulting in four fractions denominated RP1, RP2, RP3, and RP4. When these fractions were submitted to N-terminal sequencing, the comparative analysis in databanks revealed homology for two of these peptides, isolated from fractions RP3 and RP4, with sequences of proteinase inhibitors and 2S albumins, respectively. The F3 fraction, rich in peptides, inhibited the growth of yeasts *Saccharomyces cerevisiae*, *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Pichia membranifaciens*, *Kluyveromyces marxiannus*, and *Candida guilliermondii*. The RP3 and RP4 fractions showed high inhibitory activity against the growth of the yeast *S. cerevisiae*. The F3 fraction was also able to inhibit glucose-stimulated acidification of the medium by yeast cells of *S. cerevisiae* and to cause several morphological changes in different yeasts, such as cell wall disorganization, bud formation, and the formation of pseudohyphae.

18.4.3 Importance in Taxonomic Studies

Analysis of endosperm seed proteins by HPLC in many horticultural species allowed the differentiation among species, cultivars, and androgenetic lines of crop plants belonging to different botanical families [94–107].

HPLC analysis of seed storage proteins has turned out to be very useful in differentiation of landraces and/or accessions in ecotypes of horticultural crops, as, for example, the tomato (*Solanum lycopersicum* L.). For this purpose we have studied two small tomato ecotypes cultivated in the Campania (region in southern Italy): "Corbarino" and "Vesuviano" ecotypes. Several accessions belonging to the two ecotypes exist, and they probably were derived from some old canning tomato varieties, such as "Principe Borghese," "Lampadina," "Fiaschella," "Re Umberto," and so on,

through breeding and selection activity carried out by local growers both at Corbara, a village of the Salerno province at the foot of Lattari Mountains, and near Vesuvio's slopes. The accessions of "Corbarino" ecotype were mainly suitable for industrial processing and/or for fresh consumption, whereas the accessions of "Vesuviano" ecotype were used for cluster storage (named "piennoli, ceppe o spunzilli") and family consumption during the winter season or for homemade preserves. In the last years, the high interest of the local-processing factories contributed to increases in the surface areas cultivated.

Morphophysiological and agronomical studies carried out on 26 small tomato accessions compared with five commercial cultivars exhibiting small fruits established that all the accessions belonging to the two ecotypes already described were characterized by plants having indeterminate growth and a low-fruit weight (less than 25 g). Such fruits were generally ovoidal/pear-shaped with a well-defined stylar apex in the "Vesuviano" ecotype, while they were elongated-oval without a stylar apex in the "Corbarino" ecotype. The accessions of "Corbarino" showed a higher variability for almost all the characters recorded; the two ecotypes appeared clearly distinct from the commercial small tomato varieties analyzed.

A biochemical characterization of the 26 small tomato populations and five commercial varieties was carried out based on the anion-exchange HPLC (AE-HPLC) elution profiles of the seed storage proteins. Proteins were extracted from the meal (each sample was obtained from 2 g of seed lot) by stirring for 1 h at 15°C with 1 mL of water, 0.5 M NaCl, 70% (v/v) ethanol, and 1% (v/v) β-mercaptoethanol or 0.1 N NH_4OH. These fractions correspond to those described by Osborne and Campbell [108]. The suspensions were centrifuged at 11,000 rpm for 15 min. For the same sample, the procedure was repeated three times. Sample preparation for protein content determination was carried out according to the method described by Smith and Desborough [109]. The Bradford method was used for protein quantification [110]. The chromatographic conditions and data analysis, applying the Dice coefficient, were as in Mennella et al. [107]. The elution profiles of alkali-soluble seed proteins (glutelins) obtained by AE-HPLC at a wavelength of 280 nm in a time range of 0–30 min showed the presence of 28 peaks, which allowed almost all the landraces studied to be distinguished from each other. Water-, salt-, and alcohol-soluble seed proteins did not show a sufficient degree of polymorphism to enable the differentiation of the small tomato accessions. The most significant peaks (19) for the differentiatation of the tomato landraces were eluted in a time range of 0–15 min. An additional nine peaks eluted in the time range of 15–30 min evidenced a minor degree of polymorphism (Figure 18.1).

The chromatographic analysis showed either qualitative or quantitative differences among the accessions studied. Analysis of the dendrogram obtained through NTSYS-pc software based on presence/absence data of the peaks in the chromatograms permitted 20 accessions out of 26 to

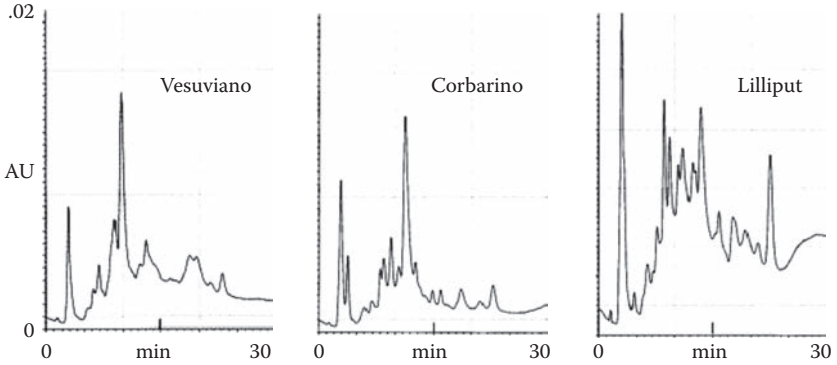

FIGURE 18.1 Anion-exchange HPLC elution profiles (time range 0–30 min) at 280 nm of seed glutelins extracted from both a "Vesuviano" and a "Corbarino" landrace in comparison with a commercial cultivar.

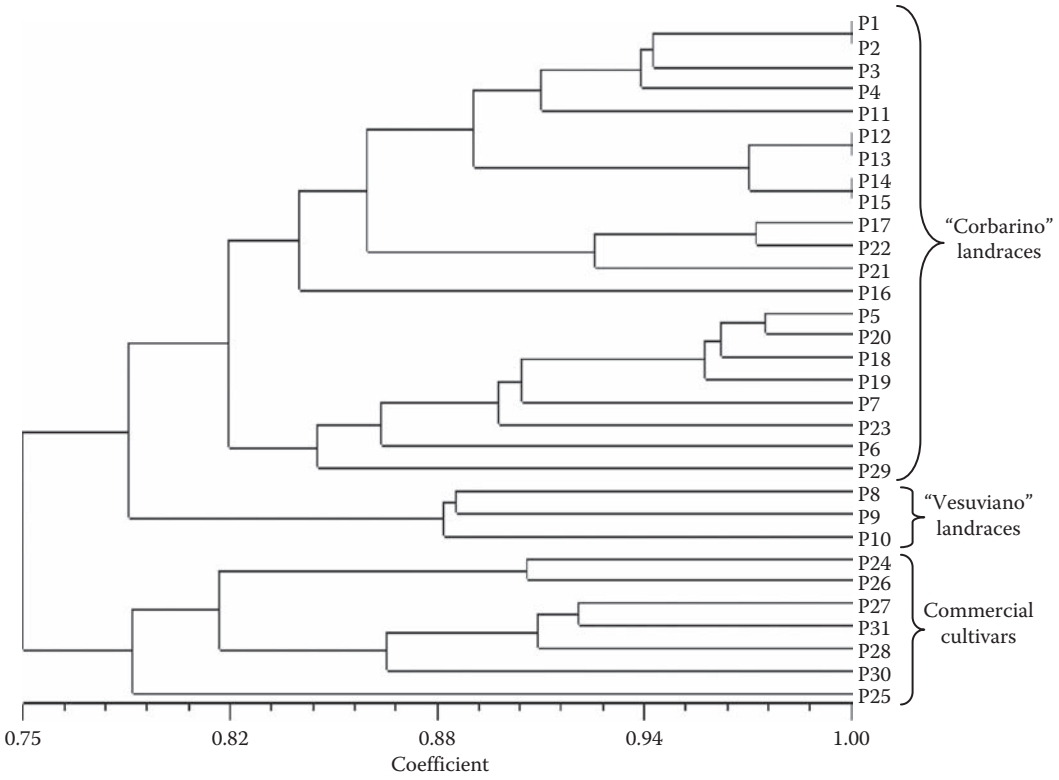

FIGURE 18.2 Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram showing relationships among the 26 small tomato landraces in comparison with the five commercial cultivars calculated by AE-HPLC data of seed glutelins.

be distinguished (Figure 18.2). The small tomato accessions referable to the "Corbarino" ecotype, except P24, P25, and P26, clustered together with a biochemical similarity of about 80% and showed a clear distinction from the commercial cultivars. The commercial industry variety "Principe Borghese" (P29), considered one of the ancestors of the "Corbarino" landraces, is included in this group. The three landraces (P8, P9, and P10) referable to the "Vesuviano" ecotype showed a biochemical similarity of about 88% and turned out to be different from those belonging to the "Corbarino" ecotype. The reference commercial varieties clustered separately (but together with the P24, P25, and P26 landraces) from the "Corbarino" and "Vesuviano" landraces (Figure 18.2). Cophenetic correlation coefficient (r) value was 0.82, which confirmed the goodness of fit.

18.4.4 Applications in Plant Breeding

One of the commonly observed responses to pathogen attack is the production of so-called pathogenesis-related (PR) proteins, many of which have antimicrobial activity [111,112]. *Fusarium oxysporum* and *Verticillium* spp. are xylem-colonizing fungi that cause important diseases in crops [113]. As far as molecular analysis of fungal wilt disease is concerned, the interaction between tomato (*Solanum lycopersicum* L.) and the host-specific *F. oxysporum* f.s. *lycopersici* is currently one of the best-studied model systems in xylem sap [47,114,115].

Resistance genes have been recently introgressed into the eggplant (*Solanum melongena* L.) gene pool from the allied *Solanum aethiopicum* gr. *Gilo* and *Solanum integrifolium* (= *Solanum aethiopicum* gr. *Aculeatum*) through the following consecutive steps: (i) somatic hybridization; (ii) anther culture from the tetraploid somatic hybrids; (iii) characterization and backcrosses of the resistant

dihaploid androgenetic plants with recurrent eggplants; and (iv) selfing to fix the resistance trait [116,117]. Here, we present the preliminary results, which aimed at understanding the resistance mechanism and proteins involved in such resistance.

We employed the radical extract because, unlike for tomatoes, it was not possible to collect the xylem sap in the eggplants used in our studies. Therefore, in order to characterize proteins involved in the early plant–pathogen interaction occurring in eggplant (or in its allied species) when infected with *F. oxysporum*, we analyzed the radical extracts from young plants of the susceptible parent *S. melongena* $1F_5(9)$ and of the resistant advanced backcrossed progeny All 96-6 × $1F_5(9)$. Plant samples were taken at 0, 8 (T0 + 8h), and 24 h (T1) after artificial inoculation by a conidia suspension of *F. oxysporum* f.sp. *melongenae* (1.5×10^6/mL).

The roots of the susceptible parent *S. melongena* $1F_5(9)$ and of the resistant backcrossed progeny All 96-6 × $1F_5(9)$ were collected, immediately frozen in liquid N_2, and stored at –80°C. Proteins were extracted from four samples: two controls (mock-inoculated) and two inoculated ones; each sample was ground to a fine powder in liquid nitrogen with a mortar and pestle. The powders were suspended in an extraction buffer consisting of 50 mM tris-HCl (pH 8.7); after centrifugation at 14,700 rpm for 15 min, the supernatant was used for protein quantification through the Bradford method [110]. One hundred microliters of sample was filtered through a 0.45 μm membrane and injected on a Shodex anionic exchange DEAE-825 HPLC column (8 × 75 mm); a Whatman anion-exchange precolumn cartridge was also used. The mobile phase was (A) 20 mM tris-HCl, pH 8.2, and (B) 20 mM tris-HCl, pH 8.2 + 0.5 M NaCl solutions. Elution was carried out according to the linear gradient: 100% A → 40% A–60% B in 40 min → 100% B in 5 min → 100% A in 5 min. Reequilibration with 100% solution A lasted 15 min prior to the next injection. Analysis conditions were a flow rate of 1 ml/min, pressure 540 psi ca, detection at 280 nm, and a temperature of 15°C. Preliminary spectrophotometric and chromatographic studies, carried out on radical extracts from resistant and susceptible genotypes, either inoculated or not, indicated that the total protein amounts, between 8 and 24 h after the inoculation, decreased only in the inoculated susceptible genotype, $1F_5(9)$, and this evidence could suggest that such a stage may be one of the most interesting ones for the plant defense response to the pathogen.

Differential chromatographic protein analyses evidenced marked differences between the susceptible and the resistant genotypes (Figure 18.3). The eluates of three successive separations were collected every minute for 15 min (range 3–18 min) and pooled. The pools were concentrated about 50 times, analyzed through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and stained by a silver staining method. The results indicated the presence, in several fractions, of proteins putatively belonging to the fungus or newly and differently synthesized by the plant in response to the *Fusarium*. Such proteins, of which the molecular weight is known, will be analyzed by MS and/or matrix assisted laser desorption ionization- time of flight (MALDI-TOF) in order to clarify their chemical identity.

18.5 PERSPECTIVES

The field of phytochemical analysis is a growing domain, not only because phytopharmaceuticals are becoming more and more important as an alternative to synthetic products, but also because new analytical approaches in genomics and proteomics open interesting perspectives in the area of plant-constituent analysis. Concerning the production of phytopharmaceuticals, the community rules implicate some restrictions, such as the characterization of leading compounds, that is, the qualitative and quantitative analysis of a representative and specific constituent found only in one plant in an extract derived from many plants. For this purpose, extraction and separation techniques have to be developed to enable qualitative and quantitative analysis. Additionally, structural elucidation based on MS, IR spectroscopy, NMR spectroscopy, and X-ray analysis has to be performed after extraction of analytes to determine the identity of compounds of interest. The field of proteomics with new analytical approaches such as two-dimensional LC hyphenated to MS opens novel

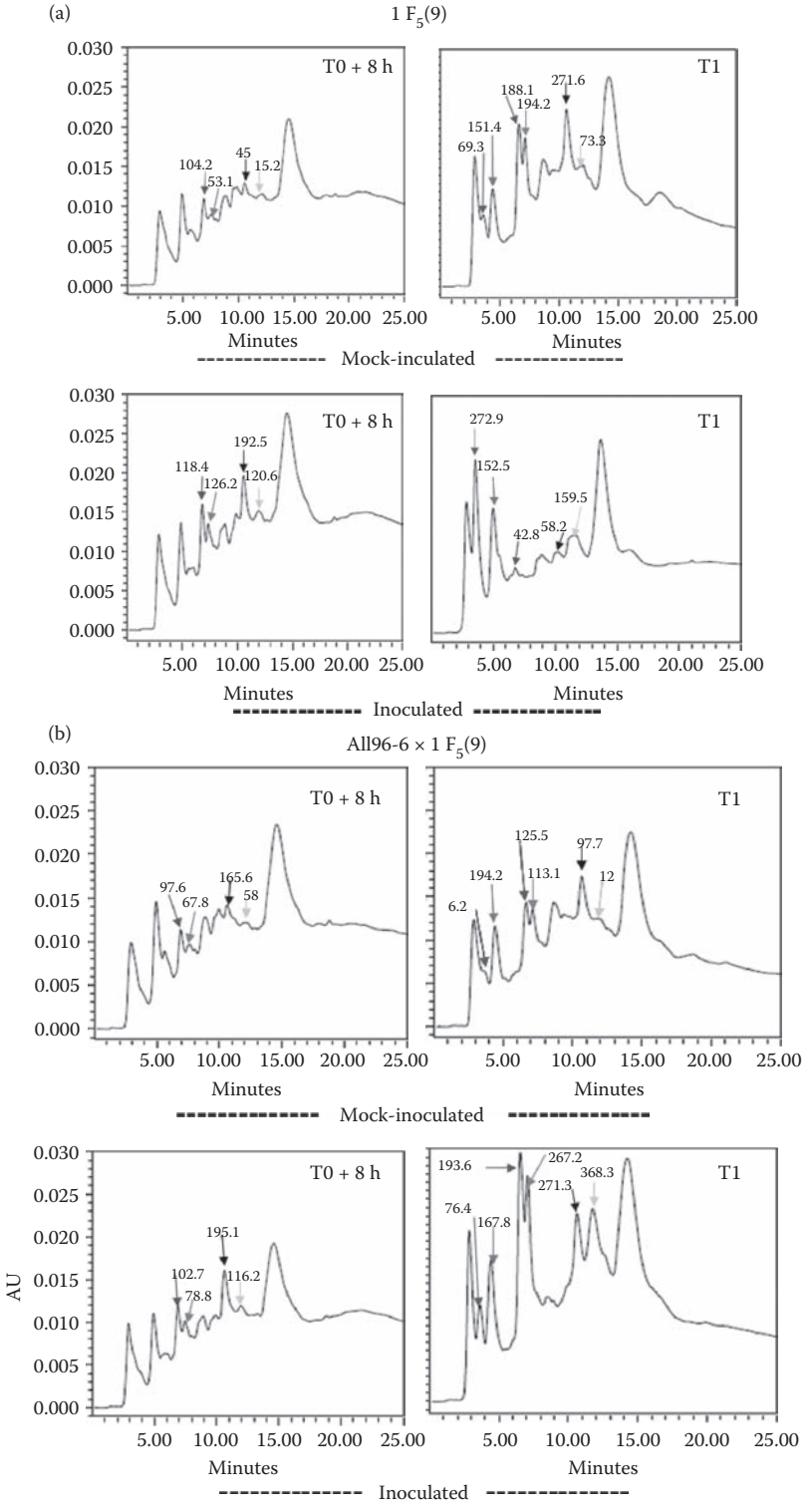

FIGURE 18.3 Anion-exchange HPLC elution profiles at 280 nm of the radical total proteins extracted, at To + 8 h and T1, from the susceptible and resistant genotypes, either mock-inoculated or inoculated.

opportunities in phytochemical analysis, such as elucidation of biosynthetic pathways or clarification of plant response.

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Secondary Metabolites — Shickimic Acid Derivatives

19 Application of HPLC in the Analysis of Phenols, Phenolic Acids, and Tannins

Tuulia Hyötyläinen and Maarit Kivilompolo

CONTENTS

19.1 INTRODUCTION

Plant phenols embrace a wide range of secondary metabolites that are synthesized from carbohydrates via the shikimate pathway. This is the biosynthetic route to the aromatic amino acids and is restricted to microorganisms and plants. Thus, phenolic compounds are ubiquitous in the plant kingdom, being found in all fruits and vegetables. The compounds are present in all parts of the

TABLE 19.1
Basic Structure of Simple Phenols, Phenolic Acids, and Tannins

Class	Structure
Simple phenolics, benzoquinones	C6
Hydroxybenzoic acids	C6-C1
Acetophenones, phenyllactic acids	C6-C2
Hydroxycinnamic acids	C6-C3
Condensed tannins	(C6-C3-C6)n

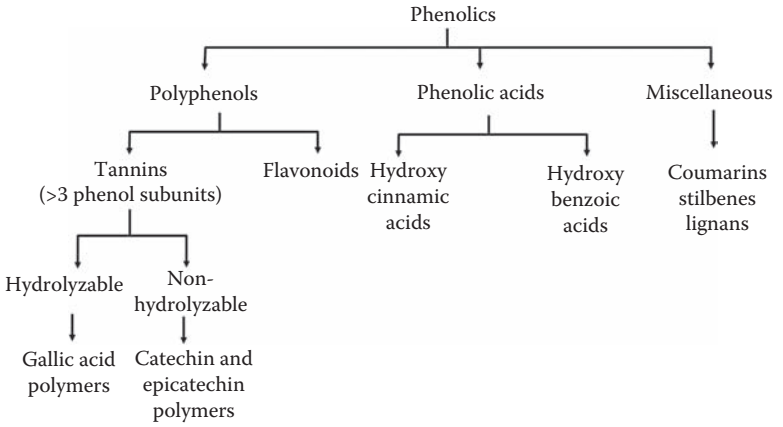


FIGURE 19.1 Plant phenolics.

plant but with quantitative distributions that vary between different tissues of the plant and within different populations of the same plant species. The phenolic compounds found in plants constitute a complex mixture, and only a small number of plants have been examined systematically for biologically active phenolic compounds. Thus, the data on phenolics of plants, fruits, and vegetables are incomplete. Over 8000 phenolic compounds with diverse structural configurations and polarities have been isolated and reported from plant sources [1], and their amount can be up to several grams per kilogram.

Phenolics display a vast variety of structures and include simple phenols, phenolic acids (both benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans, and lignins. Table 19.1 and Figure 19.1 show the most important classes of plant phenols [2]. In this chapter, simple phenols, phenolic acids, and tannins are covered.

Recent interest in phenolic compounds in plants stems from their potential protective role, through ingestion of fruits and vegetables, against oxidative-damage diseases such as coronary heart disease, stroke, and cancers. Phenolic compounds are essential for the growth and reproduction of plants and are produced as a response to defend injured plants against pathogens. The importance of phenolic compounds' antioxidant activities and their possible usage in processed foods as a natural antioxidant have reached a new high in recent years. Even though plant phenols are not always treated as real antioxidants in the literature, many in vitro studies have demonstrated the antioxidant potential of phenols as direct aqueous-phase radical scavengers and as agents capable of enhancing the resistance to oxidation of low-density lipoproteins implicated in the pathogenesis of coronary heart disease [3]. The antioxidant activity of phenolic compounds depends on the structure, in particular, the number and positions of the hydroxyl groups and the nature of substitutions on the aromatic rings.

In the determination of phenolics in plant matrices, key features are the selection of the proper sample-pretreatment technique and an efficient, sensitive, and selective separation and detection method. The type of analytes, their physicochemical properties, the type of sample matrix, and the goals of the analysis must be considered in the selection of the analytical scheme. Most phenolic compounds have polar characteristics, and therefore liquid chromatography (LC) is used in their separation.

19.2 PHYSICOCHEMICAL PROPERTIES OF PHENOLS, PHENOLIC ACIDS, AND TANNINS

Structurally, phenolic compounds comprise an aromatic ring bearing one or more hydroxyl substituents and range from simple phenolic molecules to highly polymerized compounds. Many plant phenolic compounds are polymerized into larger molecules such as the proanthocyanidins (condensed tannins) and lignins. Furthermore, phenolic acids may occur in food plants as esters or glycosides conjugated with other natural compounds such as flavonoids, alcohols, hydroxyl fatty acids, sterols, and glucosides. Interestingly, although the essential oils are often classified as terpenes, many of these volatile chemicals are actually phenolic compounds, such as eucalyptol and citronellal from clove.

The complexes of phenolics with carbohydrates, proteins, and other plant components are typically of high-molecular weight, and these complexes may be quite insoluble. Plant phenols are ionizable, with typical pK_a values ranging from 8 to 12 and oil:water partition coefficients ranging from 6×10^{-4} to 1.5 [4]. The antioxidant activity of phenolic compounds is due to their ability to scavenge free radicals, donate hydrogen atoms or electron, or chelate metal cations.

19.2.1 Phenols and Phenolic Acids

Phenols, sometimes called phenolics, are a class of chemical compounds consisting of a hydroxyl functional group (–OH) attached to an aromatic hydrocarbon group (Table 19.2). The simplest of the class is phenol (C_6H_5OH). Some phenols are germicidal and are used in formulating disinfectants. Others possess estrogenic or endocrine-disrupting activity.

The term *phenolic acid*, in general, designates phenols that possess one carboxylic acid functionality. However, when talking about plant metabolites, it refers to a distinct group of organic acids. Plant phenolic acids can be divided into several subgroups: Based on the number and arrangement of the hydroxy groups in the benzene ring they can be divided into derivatives of mono-, di-, and trihydric phenols. In addition, the majority of phenolic acids of plants are often considered to be derivatives of benzoic and cinnamic acids [5,6], as shown in Tables 19.2 and 19.3. Hydroxycinnamic acid compounds mostly occur as simple esters with hydroxy carboxylic acids or glucose. Hydroxybenzoic acid compounds are present mainly in the form of glucosides.

TABLE 19.2
Structures of Simple Phenols

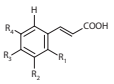
Simple phenols

Name	R_1	R_2	R_3
Catechol	OH	H	H
Hydroquinone	H	H	OH
Resorcinol	H	OH	H

TABLE 19.3
Structures of the Most Common Phenolic Acids

Hydroxybenzoic acids

Name	R_1	R_2	R_3	R_4
Benzoic acid	H	H	H	H
Gallic acid	H	OH	OH	OH
Gentisic acid	OH	H	H	OH
p-Hydroxybenzoic acid	H	H	OH	H
Protocatechuic acid	H	OH	OH	H
Salicylic acid	OH	H	H	H
Syringic acid	H	OCH_3	OH	OCH_3
Vanillic acid	H	OCH_3	OH	H
Veratric acid	H	OCH_3	OCH_3	H



Hydroxycinnamic acids

Caffeic acid	H	OH	OH	H
Cinnamic acid	H	H	H	H
o-Coumaric acid	OH	H	H	H
m-Coumaric acid	H	OH	H	H
p-Coumaric acid	H	H	OH	H
Ferulic acid	H	OCH_3	OH	H
Sinapic acid	H	OCH_3	OH	OCH_3

The acids derived from dihydric phenols are more diverse in structure. In phenolic acids derived from pyrocatechol, the carboxy group is frequently located in the *para* position to a hydroxyl. These acids are important in the vital activity of plants and are the most widespread compounds in them. They include protocatechuic, vanillic, isovanillic, caffeic, ferulic, isoferulic, and other acids. These acids may be components of alkaloids, glycosides, and lignin. Some of them have been found in plants in considerable amounts. In plants, caffeic and ferulic acids, which are most frequently regarded as derivatives of cinnamic acid, form esters with alcohols, amino alcohols, carbohydrates, and acids. Combinations of caffeic acid with malic, tartaric, shikimic, lactic, and quinic acids are known. These include chlorogenic acid and its isomers as well as chicoric acid, phaseolic acid, and rosmarinic acid.

Acids derived from hydroquinone and resorcinol form component parts of lichen acids (orsellinic, everinic, olivetolcarboxylic, and rhizoninic acids) and gentisic acid. Derivatives of pyrogallol include gallic acid, which is the structural material of tanning substances. In plants, as a rule, it is found in the form of glycosides. This group also includes metadigallic acid, metatrigallic acid, hexahydroxydiphenic acid and its lactone (ellagic acid), and syringic, eudesmic, and sinapic acids. The last mentioned, just like isoferulic acid, is found in plants in the form of an ester with choline (sinapin).

19.2.2 Tannins

Tannins are relatively high-molecular-weight compounds, and they can be subdivided into hydrolyzable and condensed tannins [7]. The former are esters of gallic acid (gallo- and

ellagitannins), while the latter (also known as proanthocyanidins) are polymers of polyhydroxyflavan-3-ol monomers (Figure 19.2). A third subdivision, the phlorotannins, are formed by the polymerization of phloroglucinol (1,3,5-trihydroxybenzene) units and are known only from brown algae [8,9].

Hydrolyzable tannins are derivatives of gallic acid that is esterified to a core polyol, and the gallyol groups may be further esterified or oxidatively cross-linked to yield more complex hydrolyzable tannins. The simplest form is the gallotannins. They are simple polygalloyl esters of glucose. The prototypical gallotannin is pentagallyoyl glucose (Figure 19.1), which has several isomers. Hydrolysis with strong acids converts gallotannins to gallic acid and the core polyol. Oxidative coupling of gallyol groups converts gallotannins to related ellagitannins. The simple ellagitannins are esters of hexahydroxydiphenic acid, which spontaneously lactonizes to ellagic acid in aqueous solutions. The ellagitannins can undergo intermolecular oxidative coupling with other hydrolyzable tannins to yield dimers.

The condensed tannins are polymers of 2–50 (or even more) flavonoid units that are joined by carbon bonds. A hydrolyzable tannin molecule contains a carbohydrate (at the center, usually d-glucose) whose hydroxyl groups are partially or totally esterified with phenolic groups such as gallic acid (gallotannins) or ellagic acid (ellagitannins). Figure 19.2 shows the structures of some typical tannins [6].

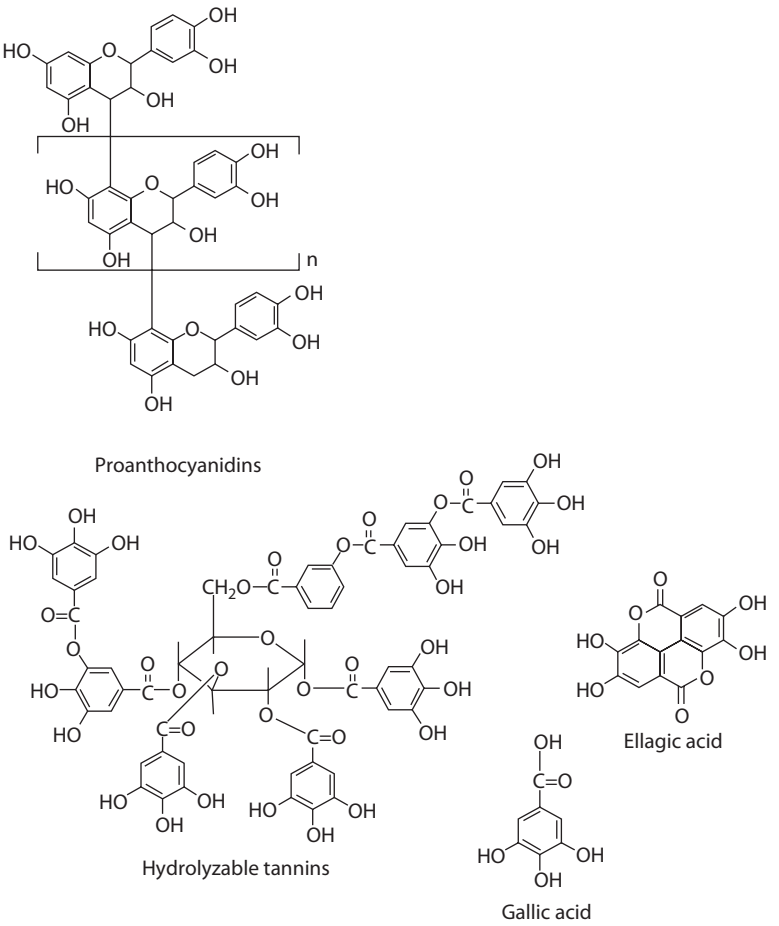


FIGURE 19.2 Chemical structures of tannins.

TABLE 19.4
Occurrence of Phenolic Acids and Tannins in Plant-Derived Samples

Compound	Found in
Hydroxybenzoic acids	Blueberries, cereals, cranberries, crowberries, black currant, raspberries, grape, raisin, plumes, apricots
Hydroxycinnamic acids	Blueberries, cereals, cranberries, oilseed
Condensed tannins	Apples, grapes, peaches, plums, mangosteens, pears, almonds, barley, grapes, tea, maize, cinnamon, cocoa, peanuts, and strawberries
Hydrolyzable tannins	Wood, bark, leaves, pomegranates, raspberries

19.3 OCCURRENCE OF PHENOLS, PHENOLIC ACIDS, AND TANNINS IN NATURAL PRODUCTS

Table 19.4 shows the general occurrence of phenolic compounds in plants. At the tissue level, there are significant qualitative and quantitative differences in phenolic content. The phenolic compounds are not uniformly distributed and may be associated with other cellular components such as cell walls, carbohydrates, or proteins. Insoluble phenolics are the components of cell walls, whereas soluble phenolics are found mainly within the plant cell vacuoles [10–13]. At the tissue level, the outer layers of plants contain higher levels of phenolics than the inner parts [10–13]. Cell wall phenolics, such as lignins and hydroxycinnamic acids, are linked to various cell components. Ferulic and *p*-coumaric acids, the major phenolic acids, may be esterified to pectins and arabinoxylans or cross-linked to cell wall polysaccharides in the form of dimers such as dehydroferulates and truxillic acid. The occurrence of phenolics in soluble, suspended, and colloidal forms and in covalent combination with cell wall components has a significant impact on their extraction. Natural hydrolyzable tannins, gallotannins and ellagitannins occur in wood, bark, leaves, and fruits. Ellagitannins are present in berries of the genus *Rubus* (raspberry, blackberry, cloudberry, arctic bramble) and *Fragaria* (strawberry), walnuts, and some other nuts [14,15]. The buds, young leaves and fresh acorns have the highest level of tannins. Proanthocyanidins can be found in, for example, apples, almonds, barley, grapes, tea, maize, cinnamon, cocoa, peanuts, and strawberries.

19.4 SAMPLE-PREPARATION METHODS

Sample preparation for the analysis of phenolic compounds in plant matrixes consists of multiple steps such as sample drying, homogenization, sieving, extraction, and preconcentration. Acid, base, or enzymatic hydrolysis is also often used to remove the sugar moieties from glycosides. Derivatization is typically not needed for HPLC analysis of phenolics. The goals of sample preparation are to isolate the analytes of interest and to improve the selectivity, detectability, reliability, accuracy, and repeatability of the analysis. Besides extraction, sample preparation often includes cleaning of complex dirty samples. Frequently, preconcentration of the analytes to a level that can be measured by the analytical method is also required.

It should be noted that there are some important distinctions between fresh and dried samples. The preliminary sample-preparation method before the extraction step can be crucial, as demonstrated in a study in which phenolics were extracted from birch leaves (*Betula pendula* Roth). In this study, it was noticed that the quantity of phenolic compounds extracted were affected by the sample-drying procedure and that, for the most reliable results, the samples needed to be analyzed immediately after collection [16].

The sample-pretreatment procedure is also challenging because many plants have a high-enzyme activity, and hence extreme care must be taken to ensure correct extraction, devoid of chemical modifications that will invariably result in artefacts. Methods of protecting the compounds from

these deteriorative processes have included the addition of antioxidants (presumably ones with higher "activity" than the compounds of interest themselves) during the extraction, the use of inert atmospheres, and absence of light. Also, hydrolysis, which is frequently used as sample-pretreatment technique in the determination of phenolic compounds in plants, can cause losses of phenolic acids [17]. Addition of ascorbic acid and ethylenediaminetetraacetic acid (EDTA) can minimize the losses during base hydrolysis [18]. Often, free phenolic compounds are extracted separately, and bound phenolic compounds are then liberated using hydrolysis followed by extraction.

19.4.1 Hydrolysis

Phenols and phenolic acids also exist as insoluble bound complexes, which are coupled to cell wall polymers through ester and glycosidic links and are not extractable by organic solvents. Bound phenolic acids are typically liberated using base hydrolysis, acid hydrolysis, or both before extraction [19]. Enzymatic treatments, such as α-amylase and cellulase, have also been used to release phenolic acids during sample preparation for detection [20]. The main step in most procedures involves base hydrolysis with NaOH ranging from 2 to 10 N and incubation times up to 16 h, sometimes under nitrogen [21]. Following base hydrolysis, acid hydrolysis is sometimes performed to liberate bound phenolics that have not yet been hydrolyzed. It has been noted that treating herbal samples with 1.2 M HCl in 50% aqueous methanol increased the yield of phenolic compounds and in vitro antioxidant activity [22]. Base or acid hydrolysis of ellagitannins yields hexahydroxydiphenic acid, which spontaneously lactonizes to ellagic acid [23]. In food samples, this reaction is commonly used in order to detect and quantitate ellagitannins as ellagic acid equivalents [24,25].

19.4.2 Extraction

No single extraction technique is suitable for all plant phenolics due to these compounds' wide diversity of chemical structures and the wide variety of sample matrixes. Phenolic compounds are known to exist as free aglycons, as conjugates with sugars or esters, or as polymers with multiple monomeric units, making the development of extraction methods challenging. In addition, the stability of phenolic compounds varies significantly; some phenolics are thermally labile, unstable, and prone to oxidation. Therefore, it is practically impossible to develop an efficient and uniform method for extraction of all phenolic compounds with a single solvent system, as the polarities of phenolic compounds vary significantly due to their conjugation status and their association with the sample matrix. Many extraction procedures incorporate the use of an antioxidant as a stabilizer; compounds that have been used for this purpose include butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ), and ascorbic acid.

Several techniques have been utilized for the extraction of phenolic compounds in plants, as can be seen from Table 19.5 [26–44]. Simple solvent extraction, also called maceration, steam extraction, Soxhlet extraction, pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), sonication-assisted extraction (SAE), and microwave-assisted extraction (MAE) have been used. Liquid–liquid extraction (LLE) and solid–liquid extraction (SLE) are still the most commonly used procedures prior to analysis of polyphenolics and simple phenolics in natural plants. As previously mentioned, pK_a values of plant phenolics vary from 8 to 12 and oil:water partition coefficients range from 6×10^{-4} to 1.5. Thus, in most solvent-based extraction methods, relatively polar solvents such as alcohols (methanol, ethanol), acetone, diethyl ether, and ethyl acetate are used. However, very polar phenolic acids (benzoic, cinnamic acids) are not efficiently extracted completely with pure organic solvents, and mixtures of alcohol–water or acetone–water are recommended. Less polar solvents (dichloromethane, chloroform, hexane, benzene) are suitable for the extraction of nonpolar extraneous compounds (waxes, oils, sterols, chlorophyll) from the plant matrix. Other factors, such as pH, temperature, sample-to-solvent volume ratio, and the number and time intervals of individual extraction steps, also play an important role in the extraction procedure. Furthermore,

TABLE 19.5
Sample Pretreatment and Liquid Chromatography (LC) Methodologies in the Analysis of Phenolic Compounds in Various Plant Samples

Sample and Compound	Pretreatment	LC Column	Eluent	Detection	Reference
Fresh plants of *T. mongolicum*	SLE with MeOH three times; evaporation, dilution, and filtration	Symmetry $C_{18,}$ 250 × 4.6 mm I.D., 5 µm	(A) 0.1% aqueous acetic acid; (B) MeOH in a gradient elution mode	UV, 254 nm, and ESI-MS in negative-ion mode	[26]
Grapeseed: catechins and proanthocyanidins (PA)	SLE with EtOH; fractionation of PA using Sephadex LH-20Exsil 100	ODS C_{18}, 250 × 4.6 mm, 5 µm) + C_{18} column guard	(A) 0.2% phosphoric acid (v/v); (B) 82% acetonitrile with 0.4% phosphoric acid; gradient	UV, ESI-MS	[27]
Apples, grapes, and beans: catechins and proanthocyanidins (PA)	SLE with 90% MeOH (apples, grapes) or 70% MeOH (beans); filtration	Inertsil ODS-2,150 × 4.6 mm, 5 µm + Opti-Guard PR C_{18} Violet	(A) 5% acetonitrile in 0.025 M phosphate buffer, pH 2.4; (B) 25% acetonitrile in 0.025 M phosphate buffer, pH 2.4	UV and fluorescence	[28]
Cocoa beans: catechins and proanthocyanidins (PA)	SLE of defatted seeds with 70% acetone (v/v), followed by extraction with 70% MeOH (v/v), fractionation of phenolics from nonphenolics on column packed with Baker octadecyl for flash chromatography	Phenomenex Luna, 250 × 4.6 mm, 5 µm	(A) Dichloromethane; (B) MeOH; (C) acetic acid–water (1:1, v/v); gradient	APCI-MS	[29]
Rhizoma Belamcandae: phenolic compounds	SAE with 70% EtOH, filtration	Alltima C_{18} ODS column, 250 × 4.6 mm ID, 5 µm + a_{18} Guard column (7.5 × 4.6 mm ID, 5 µm)	(A) Acetonitrile and (B) 0.1% TFA in water; gradient	UV, 266 nm (200 nm to 400 nm), LC/MSD trap mass spectrometer with APCI	[30]
Finger millet: free phenolic acids	Extraction with 70% EtOH, centrifugation, concentration, adjustment of pH to 2–3, extraction with ethyl acetate, evaporation, dilution	Shimpak C_{18}, 250 × 4.6 mm	Water–acetic acid–MeOH (80:5:15, v/v/v)	DAD	[31]

Berries: phenolic acids	Extraction with ethyl acetate (4 × 10 mL)	LiChroCART Purospher RP-18e column, 125 × 3 mm ID, 5 µm, + a guard column of the same material (4 × 4 mm)	20-min linear gradient of acetonitrile in 1% formic acid	UV/DAD	[32]
Flaxseed flour: defatted lignans	Extraction with 1,4-dioxane–95% EtOH (1:1, v/v), centrifugation, evaporation, alkaline hydrolysis, acidification to pH 3, removal of salt using C_{18} reversed-SPEE	Econosil RP C_{18}, 250 × 4.6 mm, 5 µm	(A) 5% acetonitrile in 0.01 M phosphate buffer, pH 2.8; (B) acetonitrile; gradient	UV/DAD, between 210 and 400 nm	[33]
White wine, grapefruit juice, and green tea infusion: phenolic acids	Dry leaves water extraction at 80°C, filtration	BEH C_8, 2.1 × 150 mm, 1.7 µm	Solvent B (acetonitrile) balanced with aqueous 7.5 mM HCOOH (solvent A); gradient	DAD + ESI-MS/MS	[34]
	SFE at 60°C and 40 MPa, CO2 + MeOH	Lichrochart RP-18, 125 × 4 mm ID, 5 µm	(A) water plus formic acid (95:5, v/v); (B) MeOH; gradient	DAD	[35]
Olive oil: phenyl alcohols, phenyl acids, secoiridoid 16 derivatives, lignans, and flavonoids	LLE with MeOH:water (80:20, v/v), rotatory evaporated, dissolved in acetonitrile, washed with *n*-hexane, evaporated to dryness, and then redissolved in MeOH	BEH C18 column, 100 x 2.1 mm ID, 1.7 µm, + VanGuardTM Pre-Column AcQuity UPLCTM BEH C18 (2.1 x 5 mm, 1.7 µm)	(A) water-acetic acid (100/0.2, v/v); (B) acetonitrile; gradient	DAD + ESI-MS	[36]
Trifolium sp.: isoflavones, phenolic acids, and clovamides		C_{18}/BEH Waters, 50 × 2.1 mm, 1.7 µm	Acetonitrile-acetic acid	UV-VIS DAD	[37]
Barley: phenolic acids	Extractions: hot water; acid hydrolysis; acid and _-amylase hydrolysis; acid and _-amylase and cellulase hydrolysis; centrifugation	Supelcosil LC-18 column, 150 × 4.6 mm, 5 µm	(A) 0.01M citrate buffer, pH 5.4, adjusted with 50% acetic acid; (B) MeOH; gradient: 0–12 min, 2–4% B; 12–20 min, 4%–13% B; 20–26 min, 13% B; 26–30 min, 13–2% B	UV, 280 nm	[20]

(*Continued*)

TABLE 19.5 (CONTINUED)
Sample Pretreatment and Liquid Chromatography (LC) Methodologies in the Analysis of Phenolic Compounds in Various Plant Samples

Sample and Compound	Pretreatment	LC Column	Eluent	Detection	Reference
Rye: phenolic acids and ferulic acid dehydrodimers	Enzymatic removal of starch; saponification: 2M NaOH, 1 h, 25°C; adjustment of pH to < 2; extraction of phenolics with ethyl acetate	LiCroCART 100 Merck RP C18 column	(A) 0.02 M phosphate buffer, pH 2.15; (B) MeOH + A (40:60); 0–50 min isocratic (75% A + 25% B); linear for 50 min to 100% B; then isocratic for 20 min (100% B)	UV, 280 nm	[38]
Herbal medicines: phenolic acids	Sonication with MeOH:water:TFA (50:50:1) for 30 min; centrifugation, filtration through 0.45 µm nylon filter	Agela XBP C18 column, 150 × 4.6 mm, 5 µm	(A) 0.02% TFA in water; (B) 0.02% TFA in MeOH; gradient: 0–5 min, 25% B; 5–10 min, 25%–30% B; 10–16 min, 30%–45% B; 16–18 min, 45% B; 18–25 min, 45–80% B; 25–30 min, 80% B; 30–40 min, 80–25% B	DAD, 254, 275, 305, and 320 nm	[39]
Oregano leaves: phenolic acids	Sequential extraction of leaves with hexane, ethyl acetate, dichloromethane, MeOH (Soxhlet, 6 h each solvent); methanolic extract was concentrated, flushed with nitrogen	RP C18 column, 250 × 4.6 mm, 10 µm	(A) water-2% CH3COOH; (B) MeOH- 2% CH3COOH; gradient: 0–60 min, 6–37% B; 60–70 min, 37–100% B; 70–90 min, 100% B; 90–105 min, 100%–6% B; 105–130 min, 6% B	UV, NMR	[40]
Eggplant: phenolic acids	Pressurized liquid extraction with MeOH, EtOH, acetone, and MeOH/water	Phenomenex Luna C18 column, 150 × 4.6 mm, 5 µm + Phenomenex C18 ODS guard column (4 × 3 mm)	(A) 0.1% aqueous formic acid; (B) MeOH; gradient: 0–50 min, 5–30% B; 50–65 min, 30% B; 65–75 min, 100% B	UV, 270 and 325 nm	[41]

Nectarines, peaches, and plums: phenolic acids, catechins, flavonols and procyanidins	Extraction with 80% MeOH containing 2mM NaF; centrifugation, filtration	Nucleosil C18, 150 × 4.6 mm, 5 µm + guard containing the same stationary phase	(A) 5% MeOH in water; (B) 12% MeOH in water; (C) 80% MeOH in water; (D) MeOH; gradient: 0–5 min, 100%A; 5–10 min, 0%–100% B in A; 10–13 min, 100% B; 13–35 min, 100%–75% B in C; 35–50 min, 75%–50% B in C; 50–52 min, 50%–0% B in C; 52–57 min, 100% C; 57–60 min, 100% D	UV, ESI-MS, negative ionization	[42]
Artichoke: phenolic acids and flavonoids	Extraction with 60% aqueous MeOH, 1 h, evaporation, dissolution in water; purification and fractionation using MeOH-activated C18 reversed-phase cartridge. Phenolic acids were eluted with 10% aqueous MeOH, and then flavonoids with MeOH	Phenomenex C18 Hydro-Synergi column, 150 × 3 mm, 4 µm + Phenomenex C18 ODS guard column (4 × 3 mm)	(A) 2% CH3COOH; (B) 0.5% CH3COOH-acetonitrile (50:50); gradient: 0–20 min, 10%–18% B; 20–30 min, 18%–24%; 30–45 min, 24%–30% B; 45–65 min, 30% B; 65–70 min, 30%–55% B; 70–75 min, 55%–100% B; 75–83 min, 100% B; 83–85 min, 100%–10% B	DAD-ESI-MS	[43]
Red raspberry: ellagic acids and flavones	Extraction with MeOH, filtration, addition of water, evaporation, semipurification of phenolics using Sep-Pak C18, filtration	Lichrocart 100 RP-18, 250 × 4 mm, 5 µm	(A) 5% formic acid in water; (B) MeOH; gradient: 0–5 min, 10%–15% B in A; 5–20 min, 15%–30% B in A; 20–35 min, 30%–50% B in A; 35–38 min, 50%–90% B in A	DAD	[44]

Note: EtOH, ethanol; LLE, ; MeOH, methanol; NaF, SFE, supercritical fluid extraction; SLE, TFA, trifluoroacetic acid.

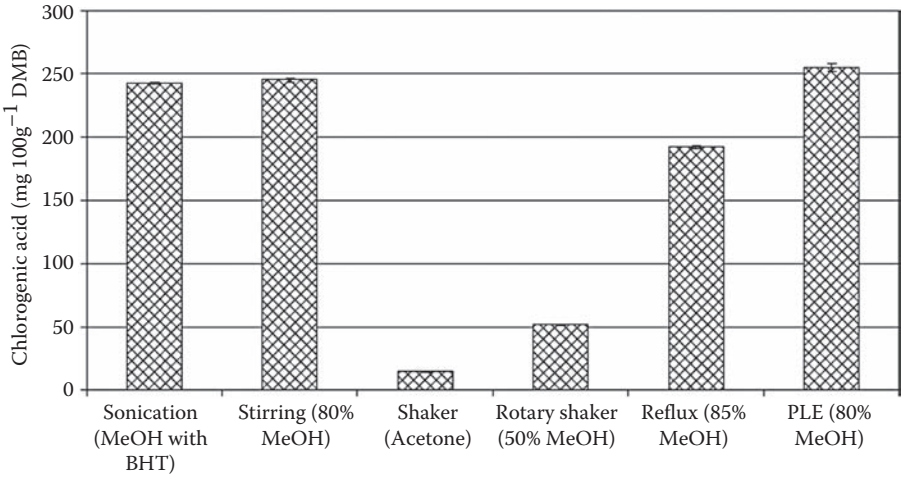


FIGURE 19.3 Comparison of extraction of chlorogenic acid (mg 100 g^{-1} dry matter basis) from an eggplant (Black Bell cultivar) sample with different extraction procedures. All extracts were analyzed by HPLC with diode array detection. MeOH, methanol; BHT, butylated hydroxytoluene. (From Luthria, D.L., *J. Sci. Food Agric*., 86, 2266–2272, 2006. With permission.)

slightly different solvents are suitable for fresh and dried samples. In the case of extractions using aqueous mixtures, the proportion of water in the extraction solvent can be lower with fresh than with dried samples. With dried materials, low-polarity solvents and ethyl acetate will simply leach the sample, whereas alcoholic solvents presumably rupture cell membranes and enhance the extraction of endocellular materials. Consequently, the relative proportion of endocellular and exocellular components may be determined by solvent choice.

The choice of the extraction method can be critical, as can be seen in Figure 19.3. In this study, six different methods were compared for the extraction of chlorogenic acid and total phenolics from eggplant [45]. Large variation in extraction efficiency was found between the different methods.

Extracts of plant materials are typically quite complex and contain interferences such as waxes, fats, terpenes, and chlorophylls. Sample cleanup requires additional steps; for example, solid-phase extraction (SPE) and fractionation based on acidity are commonly used to remove unwanted phenolics and nonphenolic substances.

19.4.2.1 Sonication-Assisted Extraction

In SAE, acoustic vibrations applied to the sample enhance the extraction. Traditionally, SAE is performed in static mode, but dynamic mode is also possible. As with other extraction techniques, dynamic extraction is advantageous in several respects: The analytes are removed as soon as they are transferred from the solid matrix to the solvent, and the continuous exposure to fresh solvent enhances the transfer of analytes from the sample matrix to the solvent. Several applications involving SAE have been published, including both static and dynamic SAE [46–49].

A recent example of the use of SAE is the extraction of phenolic compounds from Satsuma mandarin (*Citrus unshiu* Marc.) peels [46]. The contents of seven phenolic acids and two flavanone glycosides in extracts obtained by SAE were significantly higher than in extraction obtained by a conventional maceration method. In this study, increasing the extraction time and temperature increased the extraction yields. However, phenolic acids may be degraded at elevated temperatures and prolonged extraction times. Extraction for 20 min at 40°C already caused yields of *p*-coumaric acid, ferulic acid, and *p*-hydroxybenzoic acid to decrease by 48.9%, 44.2%, 48.2%, and 35.3%, respectively.

Another example of SAE use is the extraction of phenolic compounds from strawberries [47]. The extraction solvent was hydrochloric acid (0.4 M), and the extraction time was 2 min. The

method was much faster and resulted in less analyte degradation than methods such as solid–liquid, subcritical water, and MAE.

Dynamic SAE has been developed for extracting phenolic acids from basil, oregano, rosemary, sage, spearmint, and thyme, all of the Lamiaceae family [49]. A mixture of water and ethanol was used for the extraction. In this study, four variables influencing the extraction were investigated in the experimental design, namely, the flow rate, temperature, amount of ethanol, and extraction time. Of these, the temperature and extraction time had the most significant effects on the extraction yield. The optimized method was compared with three conventional extraction methods (hydrodistillation, refluxing, and liquid–solid extraction). Dynamic SAE gave the highest overall recovery for the phenolic acids.

19.4.2.2 Pressurized Liquid Extraction

In PLE, rapid extraction is performed with small volumes of conventional solvents by using high temperatures (up to 200°C) and high pressures (up to 20 kPa) to maintain the solvent in a liquid state. The use of liquid solvents at elevated temperatures and pressures enhances efficiency compared with extractions at or near room temperature and atmospheric pressure because of the enhanced solubility and mass-transfer effects and the disruption of surface equilibrium. As it uses less solvent in a shorter period of time, can be automated, and retains the sample in an oxygen- and light-free environment, it has the potential to be a powerful tool in the nutraceutical industry. The main variables that influence extraction efficiency in PLE are solvent composition, solid-to-solvent ratio, temperature, particle size distribution, and the number of extraction cycles. It has been proved that they are not degraded when 100°C is used as the extracting temperature in PLE [50].

PLE has been utilized in several studies to extract phenolic compounds from various plant matrices, such as catechin and epicatechin from tea leaves and from grape seeds [51], phenolic acids from black cohosh (*Cimicifuga racemosa*) [52], and phenolic compounds from grapes [53].

In the extraction of phenolic acids from black cohosh [52], addition of water to all solvents was found to increase the extraction by causing the plant material to swell, thus allowing the solvent to penetrate the solid particles more easily. Maximum extraction efficiency was achieved at 90°C with water–methanol (40:60, v/v) as the solvent. Particle size distribution was observed to play an important role in the extraction. There was an almost threefold increase in the extraction efficiency as the particle size decreased from greater than 2.00 mm to less than 0.25 mm ($l < 0.25$ mm).

For the extraction of phenolic compounds from grapes, a novel type of PLE method was developed in which in-cell SPE was combined with PLE [53]. LiChrolut EN was used as the solid-phase material inside the extraction chamber. Five different solvents (water, methanol, ethanol, ethyl acetate, and diethyl ether) were tested using different extraction pressures and temperatures. The best extraction efficiency was obtained with methanol at 100°C and 40 atm. Using two extraction stages with two different solvents, water and methanol, quantitative recovery for most of the assayed compounds was obtained in the second part of the extract. Only the most polar phenolic compound, gallic acid, was found distributed in both extracts. The application to real samples allows for a cleanup of the extracts. Cinnamic esters like caftaric acid and *cis*- and *trans*-coutaric acids were found only in the methanolic extract. The reproducibility of the method proved to be good.

PLE utilizing pure water has also been used in the extraction of phenolics from plants [54,55]. This technique is called pressurized hot water extraction (PHWE), also referred to as subcritical water extraction (SWE). In PHWE, the extraction is carried out using hot water, above its boiling point, that is sufficiently pressurized to maintain it in a liquid state. The extraction can be done either in a static or dynamic manner, or a combination of the two modes. The efficiency of PHWE is affected by the temperature and extraction time. It is also possible to use small quantities of organic solvents and surfactants. The polarity of water under pressure varies with temperature. The polarity of water can be tuned by the temperature; when the temperature is increased, the apparent polarity of water is decreased substantially. For relatively polar analytes, quantitative extraction can be obtained already at 100°C, whereas for less polar compounds, temperatures up to 250–300°C should be used.

PHWE has been applied for the extraction of antioxidants from rosemary leaves. Rosmarinic acid, which is a relatively polar compound, was preferentially extracted at a lower temperature (100°C), whereas carnosic acid was effectively extracted at a higher temperature (200°C) [31–32]. In PHWE, degradation of the analytes can occur due to the harsh conditions during the extraction, and this must be considered when choosing the extraction conditions. Very high temperatures should be used with caution, because excessively high temperatures may cause degradation of analytes prone to hydrolytic attack. Moreover, the selectivity of the extraction decreases at high temperatures because of the larger amounts of unwanted low-polar matrix species that are coextracted.

19.4.2.3 Microwave-Assisted Extraction

In MAE, the organic solvent and sample are subjected to radiation from a magnetron. The solvent (or the sample) must be dielectric in character. The main advantages of MAE are the fast rate of extraction due to the quick heating and elevated temperatures and also the ease of instrument operation. Elevated temperatures and the associated high mass-transfer rates are often essential when the goal is quantitative and reproducible extraction. MAE can be used in a static or a dynamic mode, or a combination of the two modes. The main factors affecting the extraction process are the power of the microwave irradiation, the liquid/solid ratio, the solvent flow rate, and the irradiation time.

The MAE conditions can be relatively harsh for labile compounds. In a recent study, the stability of several different types of phenolic compounds (benzoic acids, benzoic aldehydes, cinnamic acids, catechins, coumarins, stilbenes, and flavonols) was studied under the conditions of MAE [59]. The influence on the stability at temperatures between 50°C and 175°C was evaluated. It was noticed that all the compounds studied were stable up to 100°C, whereas at 125°C there was significant degradation of epicatechin, resveratrol, and myricetin. It was also noticed that those compounds that have a greater number of hydroxyl-type substituents are more easily degraded under the extraction conditions.

MAE has been utilized in the preparation of plant samples, such as those of *Dactylis glomerata*, *Festuca rubra* L., *Festuca ovina* L., *Bromus marginatus*, *Tilia cordata*, and *Uncaria tomentosa* [60]. MAE has also been used in the extraction of phenolic acids from *Echinacea purpurea* [61].

19.4.2.4 Supercritical Fluid Extraction

SFE provides relatively clean extracts without nonpolar interferences such as chlorophyll. Moreover, because the extraction takes place in an oxygen-free environment, the risk of degradation of labile compounds is minimal. SFE is applicable to plant samples and can also be combined with other sample-preparation techniques. All samples are usually dried before the SFE assay. However, because pure CO_2 is very nonpolar, highly polar phenolic compounds, such as flavonoids, are not extracted by 100% CO_2. The solvating power of a supercritical fluid is varied, and extraction efficiency can be markedly improved by adding organic modifiers, such as methanol.

SFE has several advantages over conventional extraction methods, such as the possibility of continuous modulation of the solvent power/selectivity, elimination of polluting organic solvents, and the reduction of postprocessing costs since there is no longer the need to eliminate solvents from the extracts [62]. A few applications of SFE have been reported for the extraction of phenolic acids or related compounds in plant matrices [63,64].

19.4.2.5 Matrix Solid-Phase Dispersion

Matrix solid-phase dispersion (MSPD) is another alternative for preparation of samples of fruits, vegetables, herbs, and other plant matrices. The technique is based on manually blending the solid or semisolid sample in a mortar with a suitable solid-phase material, such as C8- and C18-bonded silica, silica gel, sand, or Florisil [65]. The samples are usually dried with anhydrous sodium sulphate or freeze-dried before blending with the MSPD sorbent. This technique consists of distinct steps in a single process: matrix homogenization with an adsorbent phase, cellular disruption, extraction, and purification. Sample extraction and cleanup are carried out simultaneously with,

generally, good recoveries and precision. MSPD is usually done manually, although the elution can also be accomplished using, for example, PLE [65,66].

MSPD is frequently used to determine pesticides in, for example, fruits, vegetables, beverages, and foods. Although MSPD has generally been found to be simpler and faster and to require much less solvent than classical methods and has been widely used for the analysis of different analytes in plants, it has been utilized in only a few studies of phenolic compounds, such as phenolic acids [67,68].

19.4.3 Comparison of Extraction Methods

In considering any extraction technique, in addition to the effectiveness of the method, it is important to judge a range of other factors that affect the analytical scheme [69]. The most important factors are the cost of equipment, operating costs, complexity of method development, amount of organic solvent used, and level of automation. Static ultrasonic extraction is fast, but it is labor-intensive and requires a skilled operator to obtain reproducible data. Dynamic SAE avoids many of the problems of the static mode. MAE and PLE offer different advantages and disadvantages. While MAE is capable of extracting multiple samples simultaneously in a short time, additional cleanup is required to remove the sample matrix from the analyte-containing solvent, after cooling of the sample vessels. Also, MAE can be done in a dynamic mode. PLE allows multiple samples to be extracted sequentially in an automated system, but the instrumentation is relatively expensive.

Different techniques were compared for the extraction of phenolic compounds, using parsley (*Petroselinum crispum*) flakes as a model substrate [48]. This study was undertaken to address substantial variations in the extraction procedures, solvents, and conditions described in the recent literature. Five different extraction procedures (shaking, vortex mixing, SAE, stirring, and PLE) and three different solvents (methanol, ethanol, and acetone), with five different solvent-to-water ratios per solvent, were used for extraction. The study showed that the yields of phenolic compounds extracted with PLE were comparable to or better than those of the four classical extraction procedures. In another study MAE, SAE, Soxhlet, and maceration were compared in the extraction of phenolic compounds, and the antioxidative activity of the different extracts were compared [61]. The study showed that in general the MAE extracts had significantly higher antioxidative capacity than the extracts obtained with the other three methods.

In a study in which MSPD and solvent extraction combined with SPE were compared for extracting organic acids and phenolic compounds from white grapes, MSPD proved to be a simpler and faster technique, but a complete extraction of all the compounds studied could not be achieved, especially for organic acids [67]. So, although MSPD could be a useful tool to identify the phenolic and organic acid composition of white grapes, solvent extraction with SPE was preferred for quantitative recoveries of these compounds. In another study, MSPD was used for sample preparation of *Melissa officinalis* prior to LC determination of rosmarinic, caffeic, and protocatechuic acids. Different MSPD sorbents and various elution agents were tested, and the optimal extraction conditions were determined; the extraction recoveries were greater than 90% for all analytes [68].

19.4.4 Cleanup

Often, plant extracts require further cleanup before the final analysis. Many extracts contain significant quantities of interfering sugars, carbohydrates, and/or lipoidal material that potentially interfere with subsequent quantification. Several methods have been developed, including LLE, SPE, and other fractionation procedures. Also, countercurrent chromatography (CCC) has recently been explored as an alternative to LC techniques for fractionation of various classes of phenolic compounds [70,71].

LLE can be used for purification. Often the procedures require multistep extractions, as in the purification of oil seed extract, in which free and esterified phenolic acids were first extracted by

SLE using a mixture of methanol–acetone–water and the extract was further purified with LLE [17]. The free phenolics were extracted with diethyl ether, and the extract was then treated with 4 M NaOH to liberate esterified phenolic acids. Then the hydrolysate was acidified, and the liberated phenolic acids were extracted with diethyl ether. The leftover sample, after exhaustive extraction with a mixture of methanol–acetone–water, was treated with 4 M NaOH under nitrogen to liberate insoluble bound phenolic acids. The alkaline hydrolysis may lead to some degradation of hydroxycinnamic acid derivatives, but this could be prevented by the addition of 1% ascorbic acid and 10 mM EDTA [72].

Fractionation can be done using column chromatography with adsorbents such as silica gel, Amberlite XAD-2, or C18. Often, fractionation by SPE is a simpler option. Several SPE procedures have been developed. A very simple SPE method is sufficient for isolation of all acidic and basic analytes from the crude plant extract, and high recoveries are common for this simple procedure. Most frequently, the adsorbent in the SPE procedures is C18 bonded silica, and the sample solution and solvents are usually slightly acidified to prevent ionization of the phenolics, which could greatly reduce their retention. For phenolic acids, anion-exchange materials can also be utilized. Some application using molecularly imprinted materials have also been developed for the purification of phenolic plant extracts [73]. The great advantage of SPE is the possibility of combining on-line extraction with HPLC, thus achieving so-called direct sample analysis. This means that the crude extract of plant material is injected directly into this SPE-HPLC system.

An example of fractionation of phenolic compounds (polymeric procyanidins, phenolic acids, and flavonoids) from hawthorn (*Crataegus laevigata*) is presented in Figure 19.4 [74]. In this method, the phenolics in the extract were fractionated prior to HPLC analysis using column chromatography and SPE. The flavonoid fraction also contained (–)-epicatechin. The three groups of phenolics, each with clearly different UV spectra, were examined by means of HPLC–diode array detection (DAD). The polymeric procyanidins of hawthorn flowers consisted mainly of (–)-epicatechin subunits, and their mean degree of polymerization was 22.2. In this study, polymeric procyanidins were eluted from the polyamide column after fraction P3 with acetone–water in fraction P4, and fractions P1 and S1 containing other phenolic compounds were separated into flavonoid glycosides and phenolic acids using a Sep-Pak C18 plus cartridge. The phenolic acids were eluted with water from the Sep-Pak C18 cartridge, and the acids were extracted from the aqueous solution (pH 3) using ethyl acetate and diethyl ether sequentially. The adsorbed flavonoids were eluted from the Sep-Pak cartridge using methanol in fraction B. It was important that the pH of the methanol was 6.5 during the whole fractionation process. Four separate sample solutions were obtained for the analysis of phenolic compounds in hawthorn, that is, polymeric procyanidins, phenolic carboxylic acids, and flavonoid glycosides, in addition to oligomeric procyanidins. (–)-Epicatechin eluted together with the flavonoids, and the procedure thus also enabled the separation of monomeric catechins from oligomeric and polymeric procyanidins.

19.5 HPLC METHODS

The analysis of phenolic compounds is very challenging due to the great variety and reactivity of these compounds. Polyphenolics are suitable compounds for analysis using high performance liquid chromatography (HPLC) coupled with various detection techniques, such as mass spectrometry (MS), ultraviolet-visible light (UV/VIS), or nuclear magnetic resonance (NMR) spectroscopy.

19.5.1 Columns and Eluents

In the last decades, HPLC has been the analytical technique that has dominated the separation and characterization of phenolic compounds. HPLC techniques offer a unique chance to separate all analyzed components simultaneously, together with their possible derivatives or degradation

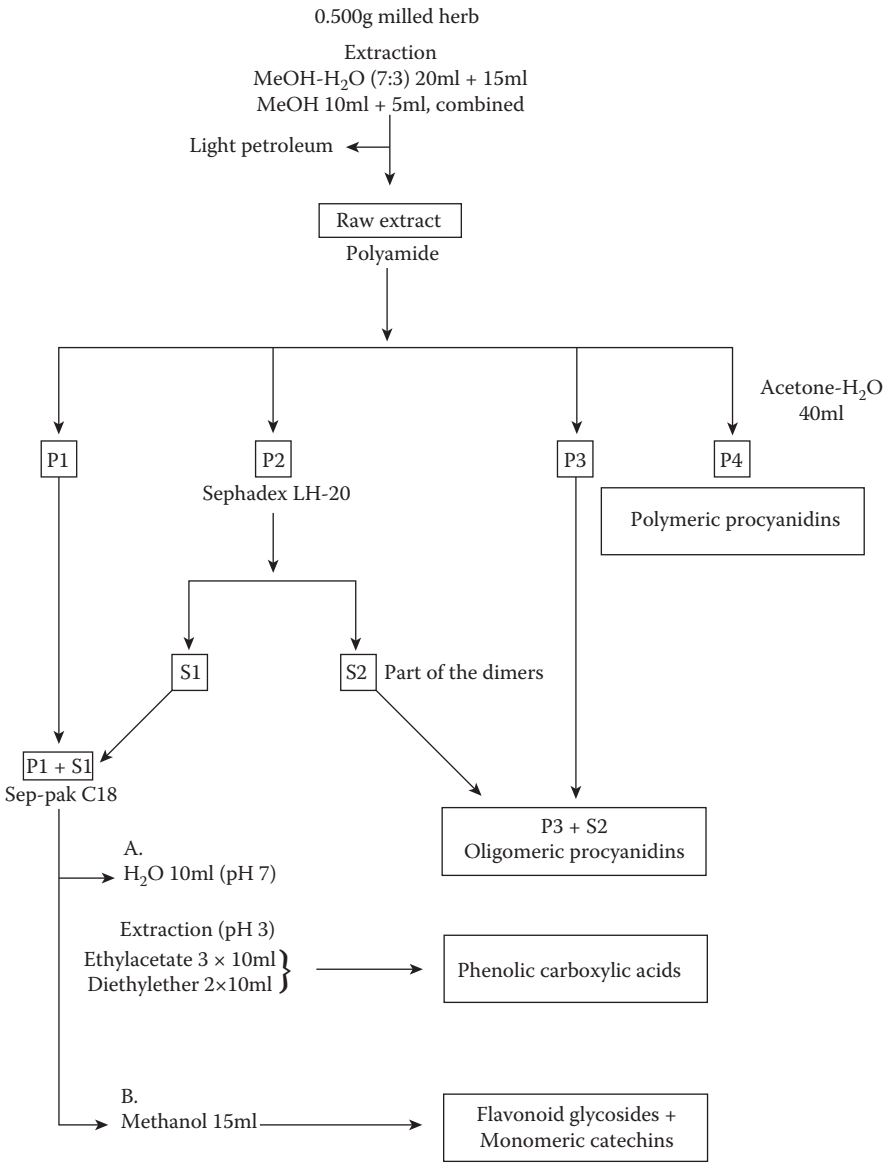


FIGURE 19.4 Fractionation procedure for phenolic compounds in hawthorn (*Crataegus laevigata*). (From Svedström, U., Vuorela, H., Kostiainen, R., Laakso, I., and Hiltunen, R., *J. Chromatogr. A*, 1112, 103–111, 2006. With permission.)

products. In many cases, they enable the determination of low concentrations of analytes in the presence of many other interfering and coeluting components. Furthermore, HPLC connected with MS detection allows reliable identification of the separated phenolic compounds.

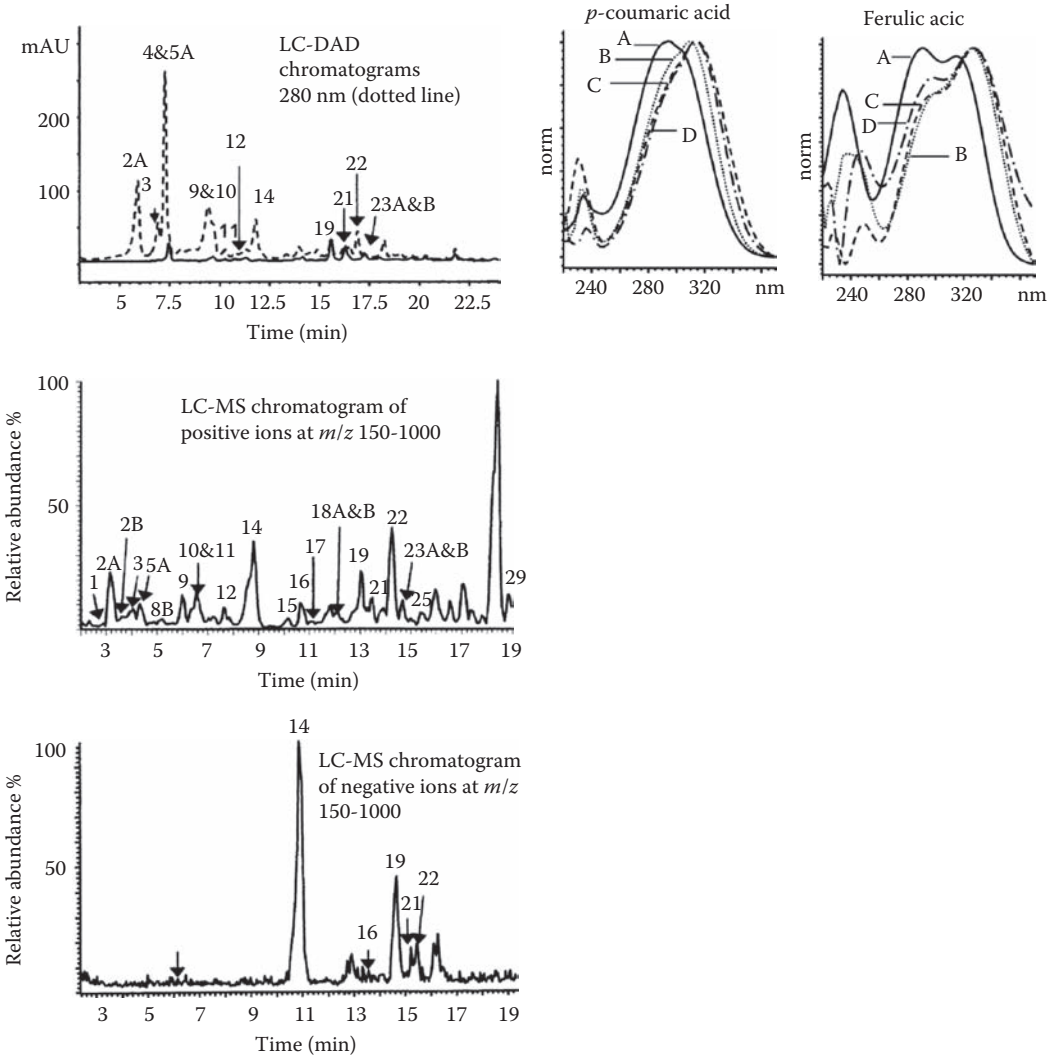
In most applications, reversed-phase (RP) LC is used in the separation of phenolic compounds. C18 phases are almost exclusively used in the separation. The column length ranges from 100 to 250 mm and the internal diameter from 2.1 to 4.6 mm. Particle sizes are in the usual range of 3–10 µm. Narrow-bore columns (2 mm ID) are recommended especially for HPLC-MS applications. The HPLC methods are listed in Table 19.5. Recently, monolithic columns and columns packed with small particles have been utilized in the analysis of phenolics in plants (see 19.6.2). By decreasing the particle size and the internal diameter of the column, analysis time can be significantly reduced.

Using columns of conventional size (length 200 mm, internal diameter 4.6 mm, particle size 5 μm), separation of phenolic acids takes about 40–60 min. Ultrafast separations of only a few minutes can be obtained with very short columns packed with sub-2-μm particles. However, these columns require an LC system capable of very high pressures (up to ca. 1000 bar). By reducing the particle size to 3.5 μm or by using fused-core particles, and reducing the column length to ca. 7–10 cm and i.d. to 2.1 mm, efficient separation of phenolic acids can be obtained in ca. 15 minutes. These columns packed with 3.5 μm particles can be operated with conventional HPLC systems, because the pressure is not excessive (<350 bar).

Gradient elution has usually been mandatory, in recognition of the complexity of the phenolic profile, although isocratic elution is still used as well. Binary systems comprising an aqueous component (acetic, formic, or phosphoric acid) and a less polar organic solvent such as acetonitrile or methanol are commonly used. In some cases, acetonitrile leads to better resolution in a shorter analysis time than methanol, and it generally gives sharper peak shapes, resulting in a higher plate number. However, methanol is often preferable to acetonitrile because of its nontoxic properties and the possibility of using higher percentages in the mobile phase. In some cases, tetrahydrofuran and 2-propanol, as less polar solvents with high elution strength, have also been used. The elution gradients for methanol usually start at 5–10% (v/v) and end at 40–100%, while the gradients for acetonitrile start at 0–10% and finish at 30–90%. In particular, separation of the more polar phenolic acids is highly dependent on the pH of the mobile phase because they are weak acids with pK_a values of about 4. Thus, a weakly acidic mobile phase will suppress ionization and enhance the separation on an RP column. In the separation of phenolic acids, the pH of the mobile phase should be about 2–2.5 with additives such as acetic, formic, or phosphoric acid or phosphate, citrate, or ammonium acetate buffer. The buffer concentrations typically vary from 5 to 50 mM. Most HPLC analyses of phenolic compounds are performed at ambient temperature, but moderately higher temperatures between 30 and 40ºC have also been recommended.

In RPLC, the retention is based on hydrophobic interactions, and the more polar compounds (e.g., phenolic acids) elute first, followed by those of decreasing polarity. Hence, the elution order is typically phenolic acids < cinnamic acids < flavonoids, although overlap of the individual members of different classes is inevitable because of the diversity of the compounds. In cinnamic and phenolic acids, polarity is increased most by hydroxy groups at the 4-position, followed by those at the 3- and 2-positions. Methoxy and acrylic groups reduce polarity and hence increase retention times.

Tannin analysis is still highly problematic. While RPLC can be used for separation of low-molecular-weight tannin polymers, it is not very useful for complex hydrolyzable tannins and condensed tannin polymers beyond tetramers [75]. Although RPLC has the ability to separate oligomers of equivalent molecular mass into their isomers, analysis of higher oligomeric proanthocyanidins (i.e., > tetramers) is not feasible, as the number of isomers increases with increasing degrees of polymerization. This effect results in a retention-time overlap for isomers with differing degrees of polymerization, causing the higher oligomers (>trimer) to coelute as a large, unresolved peak. Condensed tannins can be analyzed using the normal-phase HPLC, which, however, has much poorer resolution than RPLC. Usually, tannins are hydrolyzed before LC analysis. For example, ellagitannins are determined as ellagic acid equivalents after acid hydrolysis. HPLC methods for measuring condensed tannin composition are generally based on acid-catalyzed depolymerization of condensed tannins in the presence of a strong nucleophile, and further quantification of individual polymer subunits and calculation of average polymer length, as well as determination of total tannins. Gel-permeation chromatography (GPC) can also be utilized in the size-selective analysis of tannins. Usually, GPC is used for fractionation, and the fractions are further analyzed by MS or RPLC. An example of GPC used for the fractionation of tannins in grape seed extract is shown in Figure 19.5 [76]. The fractions were further analyzed by negative-ion ESI LC-MS (Figure 19.5B) and RPLC-MS (Figure 19.5C) prior to enzymatic hydrolysis (top) and after hydrolysis (bottom).



				Hydroxycinnamic acid derivatives				
4	6.5	234,296	326	ND	ND	ND	ND	*p*-coumaric acid 4-*O*-glucoside (std)
5A	6.9	244, 300sh, 330	342	365 (180 + 162 + 23)	185, 226, 243	341 (180 + 162 −1)	161, 179	Caffeoylhexose
6A, 7, 8A 10	7.3–9.1	236, 300sh, 314	326	349 (164 + 162 + 23)	185, 226	325 (164 + 162 −1)	145, 163	*p*-coumaroylhexose
11	9.7	236, 300sh, 314	326	349 (164 + 162 + 23)	187, 228	325 (164 + 162 −1)	145, 163, 265	*p*-coumaroylhexose
12	10.1	244, 296sh, 330	356	379 (194 + 162 + 23)	185, 226, 257	ND	ND	Feruloylhexose
20	14.9	ND	536	536 (180 + 96 + 162 + 98)	276, 438	ND	ND	Caffeic acid hexose derivative
25	17.5	236, 300sh, 314	520	520 (164 + 96 + 162 + 98)	260, 358, 422	421 (164 + 96 + 162 −1)	163	*p*-coumaric acid hexose derivative
26	17.8	234, 296, 330	452	452 (194 + 96 + 162)	290, 177	ND	ND	Ferulic acid hexose derivative

FIGURE 19.5 (A) Gel-permeation chromatography (GPC) separations of grape seed extract with a 25 × 400 mm glass column prepared with ToyoPearl HW-40S resin, methanol as the eluent at a flow rate of 1.2 mL/min, and UV detection at 280 nm. (B) Mass spectrometric (MS) analysis of compound VII-b (B2-G2). Electrospray ionization mass spectrum showing [M-H] and [M-2H]2 ions consistent with the B2-G2 structure shown (G 5 galloyl). (C) MS^2 spectrum generated by collision-induced dissociation of the *m/z* 881 ion and the structures of alternative 152 Da fragments formed by loss of a (deprotonated) galloyl group or loss of the B ring and part of the C ring of Epi via a retro-Diels-Alder reaction. (D) MS^3 spectrum from collision-induced dissociation of the *m/z* 729 ion demonstrating additional losses of *m/z* 152 fragments and interflavan bond cleavage. (E) Total ion chromatogram from LC-MS analysis of VII-b prior to enzymatic hydrolysis (top) and after hydrolysis (bottom). (From Agarwal, C., Veluri, R., Kaur, M., Chou, S.-C., Thompson, J.A., and Agarwal, R., *Carcinogenesis*, 28, 1478–1484, 2007. With permission.)

19.6 NOVEL SEPARATION SYSTEMS

In the development of sample preparation, a lot of effort has recently been put into the development of more efficient extraction methods that could replace the conventional methods, which are typically laborious and time-consuming multistep procedures requiring much manual handling of the extracts. Developing faster, more cost-effective, and environment-friendly procedures is, therefore, a pressing demand. At-line, or on-line, coupling of extraction and cleanup with the separation system is also an important goal in the development of sample-preparation methods.

The current trends in the field of LC are micro- and nano-LC, fast LC, and comprehensive two-dimensional LC. Also, novel stationary phases have been implemented. Micro- and nano-LC have, however, mainly been utilized in bioanalysis, where the sample amounts are very limited, and only some applications involving plant matrixes have been published. The development of fast-LC methods using a column packed with very small particles and very high pressures, or using monolithic columns, has been vigorous in the last few years. Another possibility to increase the separation efficiency substantially is the use of very long LC columns, either monolithic columns or columns packed with fused-core particles. With monolithic columns, the low-column backpressure makes it possible to use column lengths up to around 1 m. It is also possible to increase the resolution by using long columns packed with very small particles (<2.5 μm), instruments that are capable of pumping at very high pressures (1000 bar), and higher temperatures.

19.6.1 On-line Coupled Sample Preparation with HPLC

Most of the extraction methods are used off-line, meaning that extraction and analysis are done separately. Many of the systems could nevertheless be integrated as an on-line system, where the whole analytical procedure takes place in a closed, usually automated system. The benefits of an on-line system are the increased sensitivity and reliability because sample cleanup tends to be more effective in an on-line system [77]. The on-line systems are particularly useful in the determination of labile antioxidative compounds, because atmospheric air, moisture, and light are absent in on-line systems. Furthermore, the extraction systems utilized in the on-line systems are usually dynamic. Dynamic extraction is advantageous in many respects, since the analytes are removed as soon as they are transferred from the sample to the solvent (or fluid), and the sample is continuously exposed to fresh solvent, favoring further transfer of analytes from the sample matrix to the solvent.

Many extraction systems can be relatively easily coupled on-line with LC instrumentation [77]. SPE is readily combined with LC, and this is the most common combined system. Other extraction techniques utilized in on-line systems are LLE, SFE, PLE, MAE, SAE, and membrane-based sample pretreatment. The coupling is most commonly performed with the help of multiport valves. The SPE, LLE, and membrane-assisted techniques are best suited to liquid samples, while the other techniques are typically applied to solid samples. In on-line combinations, the volume of the extract must be kept small. Typically, it should be less than 1–2 mL, or the extract must be concentrated before the transfer to chromatography. Moreover, the solvent (or fluid) must be compatible with the following chromatographic system. In coupling with LC, the extracts should preferably be in a solvent with weak eluent strength in the LC.

A recent example of on-line coupling of extraction with LC involved the determination of phenolic acids in herbs [78]. In this study, dynamic SAE was coupled on-line with LC using a solid-phase trap as an interface between the extraction and chromatography steps. The solid-phase trap was filled with strong anion-exchange (SAX) material and served as an extra sample-cleanup step, decreasing interference from the matrix, improving the separation, and allowing UV detection. With on-line coupling the amount of sample could be smaller, and the sensitivity was increased. Moreover, the whole analysis took only 30 min, including extraction, cleanup, and analysis. The analysis time was about half that required for conventional off-line analysis. The dynamic SAE-LC system was applied to the determination of phenolic acids in basil, oregano, rosemary, sage, spearmint, and

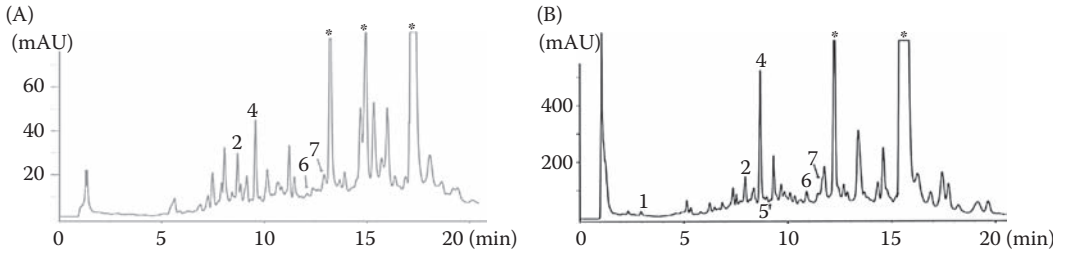


FIGURE 19.6 On-line sonification-assisted extraction (SAE)–strong anion exchange (SAX)–reversed-phase liquid chromatography (LC) analysis of phenolic acids in herbs. Chromatograms of sage samples analyzed (A) by off-line dynamic SAE and (B) by dynamic SAE-SAX-LC. Peaks: 1, gallic; 2, chlorogenic; 4, caffeic; 5, syringic; 6, *p*-coumaric; and 7, ferulic acids. The peaks marked with an asterisk have been cut off. (From Kivilompolo, M. and Hyötyläinen, T., *J. Chromatogr. A*, 1216, 892–896, 2009. With permission.)

thyme. Figure 19.6 shows chromatograms obtained with off-line sample pretreatment and with the on-line dynamic SAE-LC system. It can be clearly seen that the on-line system is more sensitive. For example, gallic and syringic acids could not be determined with the off-line procedure, but they were easily identified and quantified with the more sensitive on-line system.

19.6.2 Fast LC and Ultra High Performance Liquid Chromatography

Currently, most of the analysis methods for phenolics rely on conventional silica-based columns, and the analysis typically takes between 30 and 60 min. Even with the long analysis time, the separation power of conventional HPLC methods is not always sufficient. In many cases, MS detection can be used to distinguish between a few coeluting compounds, the number depending on the degree of similarity between the mass spectra of these compounds and on their relative concentrations in the samples. Despite the impressive potential of modern LC-MS–based systems, the chromatographic efficiency, that is, the peak capacity and resolution, of HPLC methods is still limited when using conventional columns. The recent advances in instrumentation and column manufacturing allow faster and more efficient analysis than the conventional methods allow.

The analysis time can be decreased without compromising the separation efficiency by using monolithic supports, columns packed with fused-core particles or alternatively relatively short columns packed with very small particles. The main advantage of monolithic columns lies in their excellent hydrodynamic properties, which allow reduction of backpressure and significant increase in the flow rate. The columns packed with very small particles, in contrast, generally have a very high backpressure, but the problem can be overcome by using elevated temperatures and/or pump systems capable of very high pressures.

Monolithic columns have been utilized in the separation of phenolic compounds in plant-derived samples [79–81]. A method developed for the determination of phenolic compounds in fennel (*Foeniculum vulgare*) allowed over 100 samples per day to be analyzed [80]. Chromatographic parameters such as column temperature and injection volume were found to be crucial in obtaining adequate selectivity and resolution, consequently allowing short run times. The method allowed the quantitative determination of 3-*O*-caffeoylquinic acid (3-CQA), chlorogenic acid, 4-*O*-caffeoylquinic acid (4-CQA), eriocitrin, rutin, miquelianin, 1,3-*O*-dicaffeoylquinic acid (1,3-diCQA), 1,5-*O*-dicaffeoylquinic acid (1,5-diCQA), 1,4-*O*-dicaffeoylquinic acid (1,4-diCQA), and rosmarinic acid in fennel.

The ultra high performance liquid chromatography (UPLC) system—that is, instrumentation that can operate at high pressures (ca. 1000 bar) and columns packed with very small particles (<2 μm) able to withstand these pressures — extend the limits of the separation power that has so far been achievable by commercial HPLC instrumentation [82]. UPLC has enhanced sensitivity and separation power that results in decreased analysis time and solvent consumption. Using sub-2

μm particles provides maximum efficiency, leading to column backpressures of more than 60 MPa, which are not achievable by conventional LC systems or columns. UPLC systems allow work at extreme pressures, up to 100 MPa, due to the hardware adjustments.

Several UPLC applications have been reported for the determination of phenolic compounds in plant matrices [34,36,56–57]. In the determination of 17 phenolic acids in different plants, UPLC-MS/MS was proved to reduce the analysis time significantly in comparison with conventional HPLC methods [34]. In another recent study, UPLC-MS/MS was utilized in the separation of various phenolic compounds, such as phenyl alcohols, phenyl acids, secoiridoid derivatives, lignans, and flavonoids in virgin olive oil. The retention times in UPLC were decreased three times compared to conventional HPLC, with the analysis time in UPLC being only 18 min. Furthermore, the limit of detection and limit of quantitation were generally lower in UPLC-MS/MS [36].

Another example of UPLC separation is shown in Figure 19.7. In this study, several phenolic compounds, including phenolic acids, coumarins, and flavonoids, were determined in grape wines and teas [84]. UPLC proved to have several advantages over conventional HPLC analyses. For example, in the analysis of phenolic acids, UPLC analyses were 4.6 times faster than those by HPLC. Solvent consumption was decreased by a factor of 10, and the sensitivity was about 1.7 times higher than with HPLC. Also, the repeatability of retention times and peak areas, resolution, and symmetry factor values was significantly better for UPLC.

19.6.3 Comprehensive Two-Dimensional LC

Traditional LC uses a single column, which is not sufficient for the analysis of samples with large numbers of chemical groups and compounds. According to a few recent articles, the number of analytes detected simultaneously by most HPLC methods in the analysis of phenolic compounds in plant extracts is usually around 10, with analysis time varying from 30 to 60 min [84,86]. Even the use of novel LC methods, such as UPLC, does not allow the simultaneous separation of a large number of phenolics in a single run. Comprehensive two-dimensional LC (LC × LC) is particularly suitable for the analysis of such complex samples. In LC × LC the sample is subjected to two individual separations, resulting in a tremendous increase in resolving power.

Several LC × LC applications for the analysis of phenolic compounds in plant extracts have been developed, including the characterization of traditional Chinese medicines and the analysis of antioxidative compounds in herbs, beverages, and olive oil [49,85,87–93]. In most of these applications, RPLC has been connected to another RPLC method (RPLC × RPLC), or with hydrophobic interaction chromatography (RPLC × HILIC) or with normal phase liquid chromatography (NPLC × RPLC). For the analysis of *Rhizoma chuanxiong*, a cyano column was used for the first-dimension separation and C18 as the second-dimension column [87]. Atmospheric pressure chemical ionization (APCI) was used as the interface with MS. A relatively low-eluent flow rate in the second dimension (0.7 mL/min) allowed direct introduction to the MS system. The second-dimension analysis time was long (5 min), which suggests that the system has not been, according to the definitions, truly comprehensive. The total analysis time was 225 min. A similar system with SAX as the first column was utilized for the analysis of *Flos lonicera* [87]. The second-dimension flow rate was then higher (2 mL/min), but the analysis time was still 5 min. Both methods allowed identification of several components of traditional Chinese medicines, including phenolic acids and flavonoids.

An example of LC × LC-time-of-flight (TOF)-MS being used for quantitative analysis is for phenolic acids in herb extracts and beverages [49,85,88]. In these studies, a C18 column was used for the first-dimension separation and a cyano column or C18 in ion-pair mode in the second dimension. The benefit of LC × LC for the herb analysis was clearly evident, as shown in Figure 19.8 [49]. Coeluting compounds are separated in the second dimension, making the results more reliable. For example, as can be seen from Figure 19.8E, compounds 3 and 7 coelute in the one-dimensional analysis of the spearmint extract but are well separated with the second, more polar cyano column.

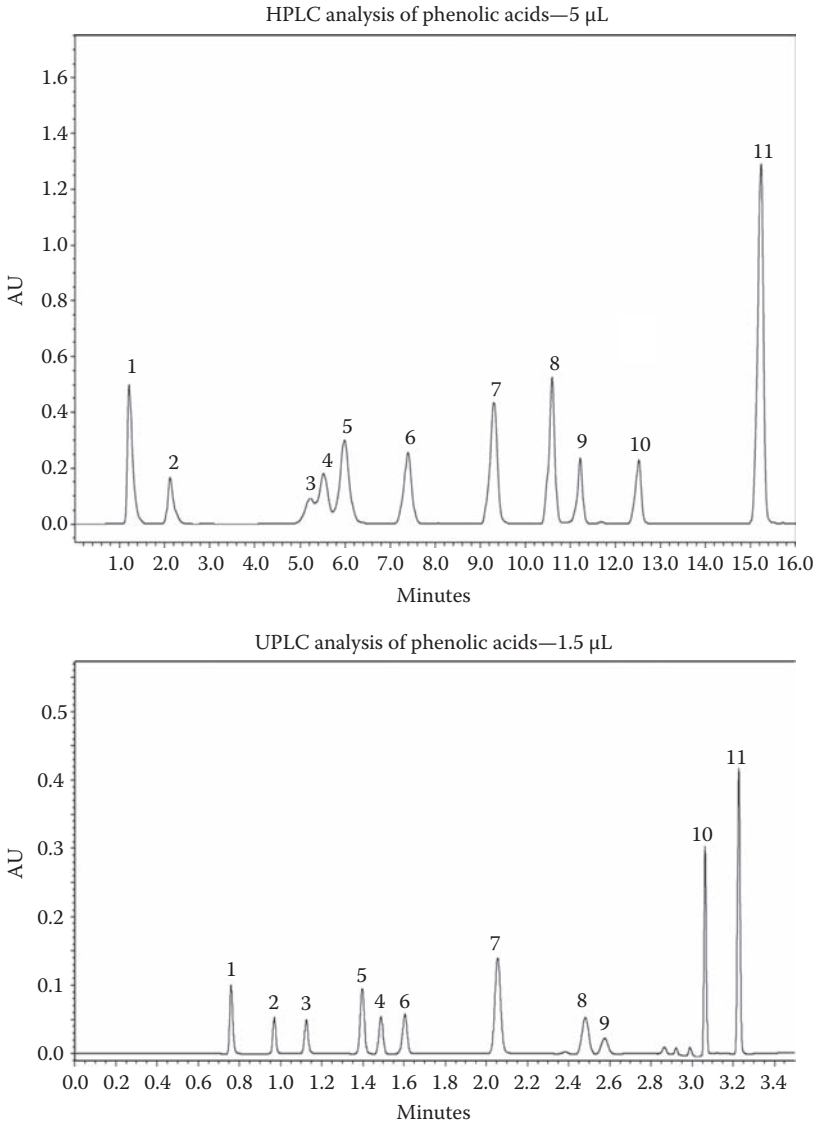


FIGURE 19.7 Comparison of HPLC and UPLC in the analysis of phenolic acids: HPLC, 0.1% formic acid–methanol, from 85:15 to 50:50 (v/v), 1.0mL min^{-1}; UPLC, 0.1% formic acid–methanol, from 88.5:11.5 to 30:70 (v/v), 0.45mL min^{-1}. Peaks 1. Gallic acid, 2. Protocateic acid, 3. Chlorogenic acid, 4. Vanillic acid, 5. Caffeic acid 6. Syringic acid, 7. p-Cumaric acid, 8. Ferulic acid, 9. Sinapic acid, 10. o-Cumaric acid, 11. Cinnamic acid. (Modified from Spáčil, Z., Nováková, L., and Solich, P., *Talanta*, 76, 189–199, 2000. With permission.)

Also, visual comparison of the samples is easy. Basil and rosemary are clearly seen to have a different chemical composition from the other herbs.

A more complex LC × LC system was used for the separation of natural phenolic antioxidants in beer and wine [91]. The first-dimension separation was done with a C18 column, and two parallel Zirconia Carbon columns were used in the second dimension. The combination of the two columns provides great differences in separation selectivity in each dimension. High-temperature isocratic separation was employed in the second dimension of the comprehensive setup, allowing improvement of the fraction transfer frequency between the two dimensions and shorter 2D separation time in comparison to the earlier method.

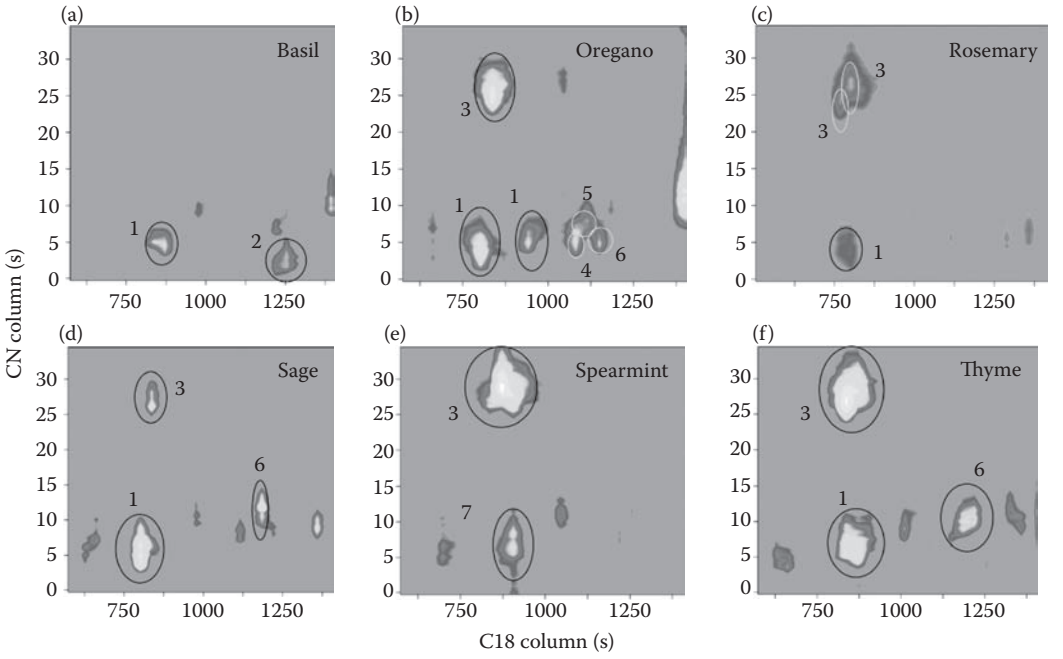


FIGURE 19.8 Comprehensive two-dimensional liquid chromatographic separation of phenolic compounds in various herbal samples. (From Kivilompolo, M. and Hyötyläinen, Y., *J. Chromatogr. A*, 1145, 155–164, 2007. With permission.)

Two LC × LC applications utilizing UPLC in the second dimension have been developed [20,93]. With UPLC, the second-dimension analysis time can be reduced without compromising the separation efficiency. The HPLC × UPLC combination was developed for the separation of phenolic and flavone natural antioxidants by using combinations of a polyethylene glycol silica microcolumn in the first dimension and a porous-shell fused-core C18 column in the second dimension, both in the reversed-phase mode [93]. Using the porous-shell C18 column in the second dimension at elevated temperature (60°C) and high pressure (480 bar) with optimized segmented profiles of the parallel gradients in the two dimensions, the overall separation time for comprehensive LC × LC was reduced to 30 min.

Another HPLC × UPLC application was for the separation of phenolic acids in herb extracts [85]. In this study, one- and two-dimensional LC systems, namely, conventional HPLC, UPLC, HPLC × HPLC, and HPLC × UPLC systems, were developed and evaluated for the separation of phenolic acids in wine and juices. In the LC × LC studies, the first-dimension separation was based on RPLC, and the second dimension was performed with ion-pair chromatography. Three different columns, namely, two short columns packed with either 2.5 μm or 1.7 μm particles and a monolithic column, were tested for the fast second-dimension separation. Interestingly, the separation efficiency of HPLC × UPLC was not as good as that of HPLC × HPLC utilizing a monolithic column for the second-dimension separation. Comparison of the HPLC × HPLC with one-dimensional separations with HPLC and UPLC showed that HPLC × HPLC gave clearly higher separation efficiency. However, UPLC analyses also provided reasonable separation for most of the target compounds, but, although the separation was clearly better than with the conventional HPLC system, baseline separation was not obtained for all compounds. In addition, in real samples, coelution of matrix compounds could not be avoided. However, in this study a relatively short UPLC column was used, and a better separation may be possible by using longer UPLC columns.

19.7 DETECTORS

UV/VIS spectroscopy and MS are the primary methods of detection employed with LC separation of phenolic compounds. The multiple conjugate bonds make phenolic compounds strong chromophores with high UV absorption. In addition, the molecular structure of the phenolic compounds makes them fairly easy to ionize by ESI and APCI. In general, sensitivity is greater with negative ionization, but this is highly compound specific. Electrochemical detection has been used with a series of voltammetric or coulometric detectors but has had limited use. Fluorescence detection has also had limited application since few phenolic compounds fluoresce. The disadvantage of HPLC-DAD is that the UV spectra of phenolic compounds are often very similar, and the possibility of unambiguous identification does not exist.

Absorption spectra can be combined with retention parameters to potentially identify unknown compounds, to measure purity of the elution band in question, and to perform quantitative sample analysis. However, in the identification, MS detection is more reliable and powerful, particularly for unresolved mixtures of phenolics. In quantitation, LC-UV/VIS is more reliable than LC-MS, if the separation is satisfactory. Table 19.6 lists some relevant detector properties of phenolic acids.

19.7.1 UV/VIS

Phenolic compounds absorb well in the UV range, and UV detection is therefore a convenient detection method for these compounds. Phenolic compounds exhibit a higher or lower absorption in the UV or UV/VIS region depending on the intrinsic existence of conjugated double and aromatic bonds. Phenolic acids with the benzoic acid carbon framework have their maxima in the 200–290 nm range. The only exception is gentisic acid, which has an absorbance that extends to 355 nm. The cinnamate derivatives, due to the additional conjugation, show a broad absorbance band from 270 to 360 nm.

Detection at 280 nm is most generally used for the simultaneous separation of mixtures of phenolic acids. However, single-wavelength detection is usually not sufficient for reliable detection of

TABLE 19.6
Typical Ultraviolet Absorbance Maxima, Mass Spectrometric (MS) Fragmentation Pattern, and Fluorescence and Electrochemical Properties of Selected Phenolic Acids

Compound	MW	[*M*-H]- (Frag. MS2*m/z*)	UV Band (nm)
Gallic acid	170	169 (125)	272
Protocatechuic acid	154	153 (109)	260 (max), 294
Vanillic acid	168	167 (123)	260 (max), 294
Syringic acid	198	197 (182, 153)	276
p-Hydroxybenzoic acid	138	137 (93)	256
Salicylic acid	138	137 (93)	276
Gentisic acid	154	153 (109)	326 (max), 300
Caffeic acid	180	179 (135)	324 (max), 296
p-Coumaric acid	164	163 (119)	310
Sinapic acid	224	223 (208, 179, 149)	324
Ferulic acid	194	193 (134, 149, 179)	324 (max), 296
trans-Cinnamic acid	148	147	278
Chlorogenic acid	354	353 (190)	326 (max), 300

phenolics in a complex plant matrix. DAD is a more reliable option for identification. Moreover, DAD spectra can be used to estimate the purity of analytes.

19.7.2 MS

Because polyphenolic compounds are usually found as complex mixtures in plants, hyphenated techniques such as LC-MS are typically needed for accurate identification of plant phenolics. LC-MS with different ionization modes represents a rapid and reliable technique for analyzing phenolic substances. In the analysis of phenolic compounds, most of the system utilize ESI or APCI interfaces [94–97]. In some cases the coupled technique can afford a full on-line structural analysis involving no time-consuming isolation process. Soft ionization with ESI-MS provides the molecular masses of chromatographically separated molecules, and tandem mass spectroscopy (MS/MS) provides extra structural details for thermally labile, nonvolatile polar phenolic compounds. With TOF-MS, accurate masses can be obtained. Ionization may be performed in the positive- and/or negative-ion mode. In the negative-ionization mode, acidic hydroxybenzoic and hydroxycinnamic acids deprotonate easily [63–64], whereas in the positive-ionization mode, they form adducts with the cations in the sample or mobile phase, for example, sodium ions [98–101].

Most LC-MS methods for the separation of phenolic compounds in plant-related samples are based on RPLC separation, mainly using C18 columns and aqueous mobile phases. It should be noted that the quantitative analysis in LC-MS can be significantly affected by matrix effects, that is, coeluting ionic and ionizable constituents of the sample and the sample matrix, which can show major effects on the ionization yield of the analyte. Also, mobile-phase additives, such as buffers or ion-pairing reagents, can severely influence signal intensity.

Several LC-MS applications have been reported for the analysis of phenolic compounds in plants. In a recent study, phenolic compounds from the lingonberry (*Vaccinium vitis-idaea*) were identified using LC-TOF-MS, LC-MS/MS, and NMR experiments [105]. The compounds were extracted from the plant material using SAE with methanol as the solvent. The extract was further purified using SPE and preparative LC techniques. In this study, 28 phenolic compounds were characterized from the berries, leaves, and stems of lingonberry. The identified compounds included flavonols, anthocyanidins, catechins and their glycosides, and different caffeoyl and ferulic acid conjugates. In another study, LC-ESI-MS was utilized in qualitative analysis of the phenolic fraction of *Y. gloriosa*, while LC-ESI-MS/MS was used in quantitative studies. LC/ESIMS/MS was used in multiple reaction monitoring (MRM) mode [106].

In a recent study, the phenolics in *Taraxacum mongolicum*, a traditional Chinese medicine, were characterized by LC–DAD–radical-scavenging detection–ESI-MS [26]. For the sample pretreatment, extraction was done with methanol, followed by cleanup utilizing preparative HPLC and Sephadex LH-20. The purified compounds were sampled to an off-line NMR spectrometer to acquire NMR spectra. The structure of the active compounds was elucidated by UV, ESI-MS, and NMR spectral data. Thirty-two radical-scavenging compounds, including 16 flavonoid derivatives, 10 phenylpropanoid derivatives, and 6 benzoic acid derivatives, were screened, isolated, and identified. Among them, seventeen compounds including three new compounds were first isolated from *Taraxacum* genus. Caffeic acid **(6)**, isoetin-7-*O*-β-d-glucopyranosyl-2′-*O*-α-l-arabinopyranoside **(9)**, isoetin-7-*O*-β-d-glucopyranosyl-2′-*O*-α-d-glucopyranoside **(10)**, and isoetin-7-*O*-β-d-glucopyranosyl-2′-*O*-β-d-xyloypyranoside **(12)** were found to be the major metabolites in *T. mongolicum*.

RPLC-TOF-MS is also an attractive tool in the identification of phenolics in complex plant matrices due to its high-resolving power, accurate mass measurement capability, and full spectral sensitivity. With TOF-MS, a narrow mass window of 0.01 Da can be used, unlike in conventional MS, which has a typical mass window of 1 Da. An example of utilization of RPLC-TOFMS is

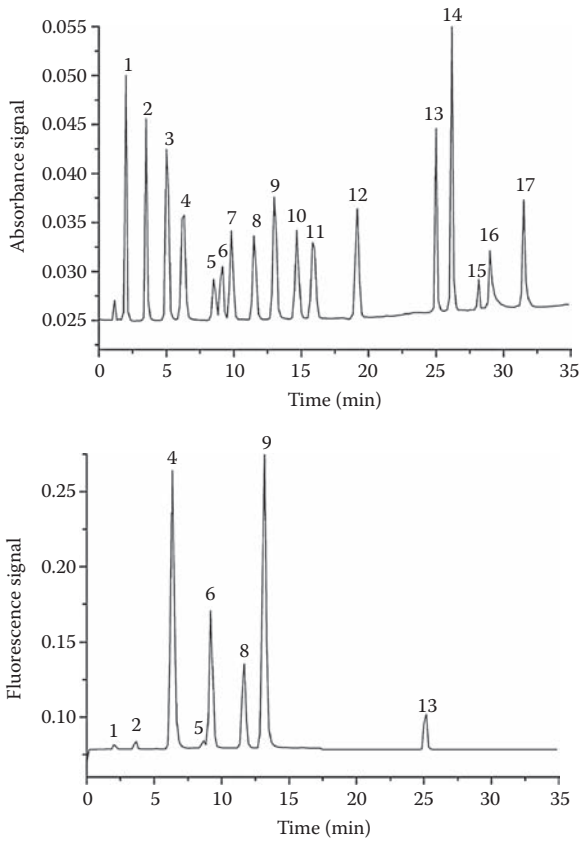
the determination of major phenolic compounds in Danggui Buxue Tang (DBT) preparation [107]. In this study, also data from DAD was utilized in the identification. For example, one unidentified compound had maximum UV absorption at 217, 235, 324 with a shoulder peak at 290 nm. In positive ion mode with a moderate fragmentor voltage, it produced dominant protonated ion $[M+H]^+$ at m/z 195.0658 ($C_{10}H_{11}O_4$) and a minor sodium adduct ion $[M+Na]^+$ at m/z 217.0482. With the fragmentor voltage increased to 250 V, several characteristic fragment ions were obtained. The base peak at m/z 177.0541 ($C_{10}H_9O_3$) is formed by loss of one molecule of water from the protonated ion. Another major fragment ion at m/z 149.0596 ($C_9H_9O_2$) is formed by successive or simultaneous loss of a molecule of CO from 177.0541, and some minor peaks at m/z 134.0365 ($C_8H_6O_2$) and 117.0332 (C_8H_5O) result from loss of a methyl group and CH_3OH from 149.0596, respectively. In addition, loss of 32 Da (CH_3OH) from 177.0541 leads to the formation of the ion at m/z 145.0280 ($C_9H_5O_2$). Consequently, the unidentified compound was unequivocally identified as ferulic acid and further confirmed by comparison with the authentic standard.

In two recent studies, LC-MS has been utilized in the analysis of tannins in different types of samples. LC-ESI-MS in negative-ion mode was utilized in the characterization of tannin-enriched extracts from raspberry, cloudberry, and strawberry [105]. To obtain comprehensive data, two scan events were used; a full scan analysis followed by data-dependent MS/MS of the most intense ions. The raspberry and cloudberry extracts contained a similar mixture of identifiable ellagitannin components and ellagic acid. However, the strawberry extract contained a complex mixture of ellagitannin and proanthocyanidin components that could not be adequately resolved to allow identification of individual peaks. In another study, extractable tannins were analysed by LC-ESI-MS in two oak species, North American white oak (*Quercus alba*) and European red oak (*Quercus robur*) [109]. The sample pretreatment included extraction with methanol–water (80:20, v/v) at room temperature for 24 h and removal of methanol by vacuum distillation. Finally, the aqueous residue was extracted with diethyl ether. The compounds identified from the oak samples mainly included various glucose gallic and ellagic acid esters. The structures were partially determined, and they included grandinin/roburin E, castalagin/vescalagin, gallic acid, valoneic acid bilactone, monogalloyl glucose, digalloyl glucose, trigalloyl glucose, ellagic acid rhamnose, quercitrin, and ellagic acid.

19.7.3 Other Detectors

In addition to UV and MS detectors, fluorescence (FL) and electrochemical detectors (ED) can also be used in the determination of phenolic compounds [110–118]. ED and FL detectors are very sensitive and selective; however, they do not respond to all phenolic compounds. Therefore, FL detectors and ED are usually used in series with UV or MS detection. For example, the use of UV and FL detectors in series allows discrimination of fluorescent and nonfluorescent overlapping peaks, as shown in Figure 19.9 [115]. Chemiluminescence and NMR detection can also be used [119].

An example of the use of several detectors is for the study of the phenolic composition of wines using HPLC with MS and DAD, ED, and FL detectors [120]. For resveratrol, piceid, gallic acid, protocatechuic acid, catechin, and quercetin, fluorescence and electrochemical properties were used as complementary or alternative methods of detection. LC-MS using APCI in negative mode proved to be a valuable tool for qualitative analysis of phenolic acids, such as gallic, protocatechuic, and caffeic acids and catechin, epicatechin, piceid, and quercetin glycosides. The use of tandem detectors (LC-DAD-FL-ED) allowed selective detection of compounds such as gallic acid, protocatechuic acid, catechin, and quercetin with ED, while DAD and FL detectors allowed discrimination of fluorescent and nonfluorescent overlapping peaks such as resveratrol and piceid.



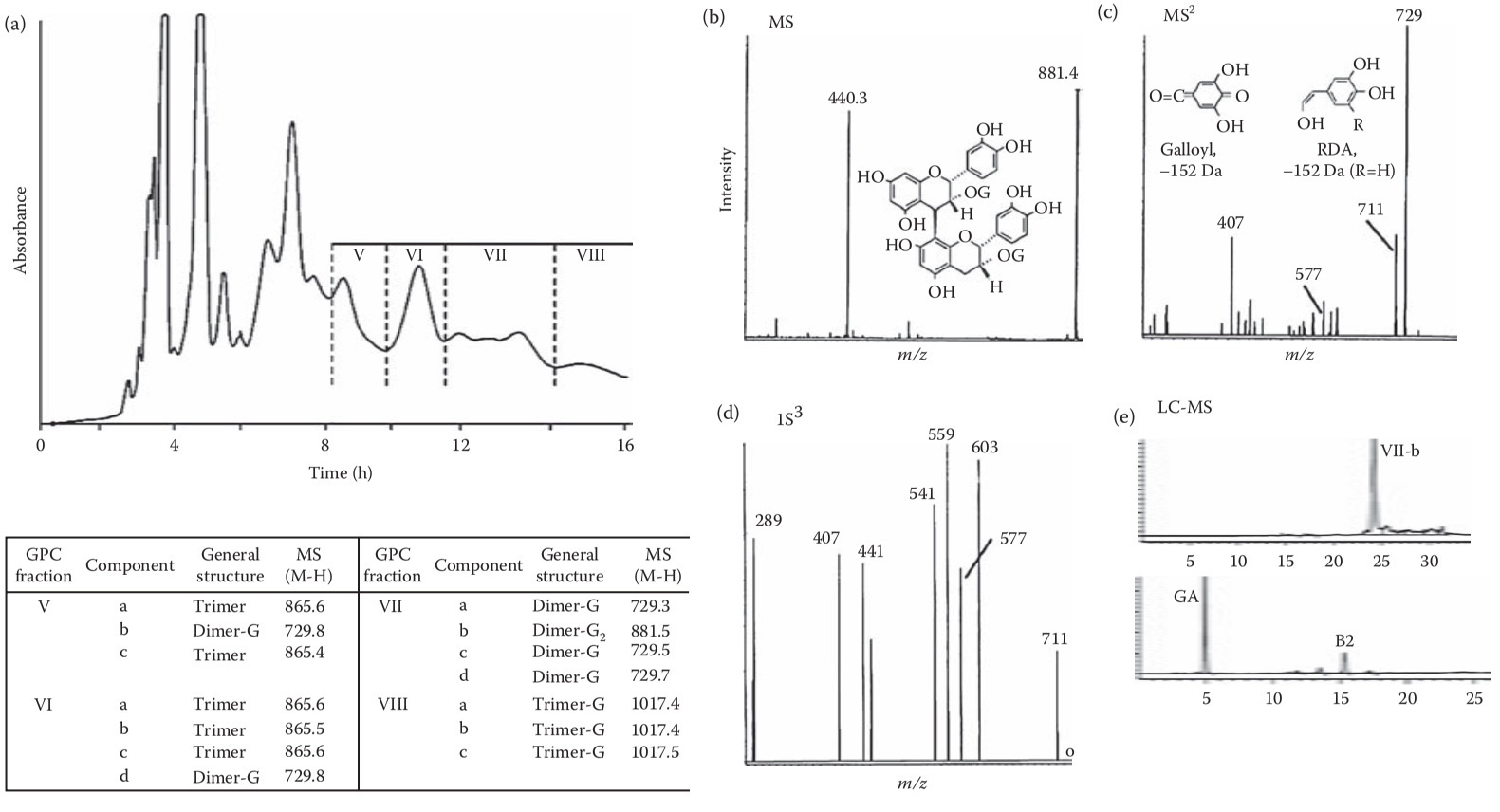
No.	Compound	Excitation (nm)	Emission (nm)
1	Gallic acid	278	366
2	Protocatechuic acid	270	358
3	Protocatechuicaldehyde	265	360
4	(+)–Catechine	278	360
5	2,5-Dihydroxybenzaldehyde	278	360
6	Vanillic acid	278	360
7	Caffeic acid	262	426
8	Syringic acid	278	360
9	(–)-Epicatechin	278	360
10	Syringaldehyde	260	422
11	*p*-Coumaric acid	260	422
12	Ferulic acid	260	422
13	*trans*-Resveratrol	330	374
14	Myricetin	268	370
15	Quercitrin	260	426
16	Quercetin	264	420
17	Kaempferol	268	422

FIGURE 19.9 Chromatograms of a standard mixture of polyphenolics determined from replicate assays of wine spiked with compounds using (A) absorbance detection (λ 280 nm), (B) known concentrations of polyphenolic compounds fluorescence detection (λ_{ex} 278 nm and λ_{em} 360 nm over 17.5 λ_{em} min and λ_{ex} 330 nm and λ_{em} 374 nm for 16.5 min). (From Rodriguez-Delgado, M.A., Malovaná, S., Pérez, J.P., Borges, T., and Montelongo, F.J.G., *J. Chromatogr. A*, 912, 249, 2001. With permission.)

19.8 QUANTITATIVE AND QUALITATIVE ANALYSIS

The role of sample preparation is key in the development of a quantitative method for the analysis of phenolic compounds in plant matrices. The most reliable results are obtained with fresh samples. Particularly with labile compounds, on-line methods in which the sample preparation is integrated with the separation system are highly useful. In off-line methods, the stability of the samples during the sample-preparation steps must be ensured, for example, by addition of antioxidants, the use of inert atmospheres during sample treatment, and protection of the sample from light.

For reliable quantitative and qualitative determination, sufficient separation of analytes of interest and other interfering compounds must be obtained during the LC step. In many cases, MS detection can be used in distinguishing between coeluting compounds. However, with severe coelution, identification even with MS can be problematic, depending on the degree of similarity between the mass spectra of these compounds and on their relative concentrations in the samples. In these cases, MS/MS detection is a useful option. However, even when MS and MS/MS modes are available, identification is not an easy task because universal libraries are not available. Furthermore, many fragmentation spectras of phenolic acids are very similar, making the identification even more challenging. It should also be noted that in quantitation, UV detection is often more reliable than MS detection, due to matrix effects that are typical for MS detection of complex samples. Of course,



GPC fraction	Component	General structure	MS (M-H)	GPC fraction	Component	General structure	MS (M-H)
V	a	Trimer	865.6	VII	a	Dimer-G	729.3
	b	Dimer-G	729.8		b	Dimer-G_2	881.5
	c	Trimer	865.4		c	Dimer-G	729.5
					d	Dimer-G	729.7
VI	a	Trimer	865.6	VIII	a	Trimer-G	1017.4
	b	Trimer	865.5		b	Trimer-G	1017.4
	c	Trimer	865.6		c	Trimer-G	1017.5
	d	Dimer-G	729.8				

FIGURE 19.10 Chromatograms of liquid chromatography-diode-array detection and liquid chromatography-mass spectrometry in positive- and negative-ionization modes and UV-visible spectra of (A) 4-*O*-glucoside, (B) aglycon, (C) hexose ester, and (D) a less polar hexose derivative of *p*-coumaric and ferulic acids in high performance liquid chromatography-diode-array detection. Sample: White currant extract. (From Määttä, K., Kamla-Eldin, A., and Törrönen, A.R., *J. Agric. Food Chem.*, 51, 6736–6744, 2003. With permission.)

reliable quantitation with a UV detector requires sufficient separation of the target compounds from other compounds.

Figure 19.10 shows an example of identification of phenolics by use of HPLC retention data, UV spectra, and MS and MS/MS spectras [97]. LC-MS and MS/MS were used in both positive- and negative-ionization modes in order to obtain more information on the structural features of the conjugated forms of the phenolic compounds. UV/VIS spectrometry proved to be a valuable tool for the identification of the class of phenolic compounds, whereas MS and MS/MS fragmentation data were useful for further structural characterization. For example, the UV/VIS spectrum and the retention time of peak 4 matched that of *p*-coumaric acid 4-*O*-glucoside. The glycosylation of the hydroxyl group in *p*-coumaric acid caused a hypsochromic shift (from 310 to 296 nm) and the disappearance of the typical spectral feature of the aglycon. UV/VIS spectral identification allowed the assignment of peaks 6A, 7, 8A, 10, 11, and 25 as *p*-coumaric acid derivatives and peaks 5A, 12, 20, and 26 as caffeic and/or ferulic acid derivatives. These peaks have experienced a shift of the major absorption maximum from 310 to 314 nm (*p*-coumaric acid) and from 326 to 330 nm (caffeic and ferulic acid) compared to the respective free standards. These bathochromic shifts suggest that the substituents are esterified to the carboxylic functions of the hydroxycinnamic acids. The positive MS/MS fragmentation spectra of the hydroxycinnamic acid esters consisted of the sodium adducts of hexose, as did those of the hydroxybenzoic acid esters. In the negative-ion LC-MS, caffeoylhexose (peak 5A) and *p*-coumaroylhexose (peak 10) exhibited deprotonated ions with very low responses at *m*/*z* 341 and 325, respectively. In the MS/MS spectra, these two compounds displayed ions of *p*-coumaric and caffeic acids at *m*/*z* 163 and 179, after elimination of a hexose moiety, and at *m*/*z* 161 and 145, after the subsequent elimination of water. In addition to these compounds, several other phenolic compounds could be identified, such as flavonol glycosides and anthocyanin pigments, cyanidin 3-*O*-sambubioside and quercetin hexosidemalonate, and flavonol hexoside-malonates.

For more reliable identification, LC × LC is a useful option as it produces two independent retention times for each peak, and this is very useful for more reliable identification. An example of utilization of LC × LC-MS in qualitative and quantitative analysis is the determination of phenolic acids in herbs. Identification of the compounds from their mass spectra alone was difficult, but chemical characterization of the analytes based on their location in the 2D retention plane and their spectra is feasible. This feature is demonstrated in Figure 19.8 for the identification of unknown compounds in spearmint extract. In this application, the first-dimension separation was done with a C18 column, and the retention was thus based on hydrophobic interactions. The second-dimension separation was done with a cyano column, using aqueous normal-phase mode, and the retention was mainly based on polar interactions. For example, compound 7 in Figure 19.8E in the spearmint extract has an *m*/*z* value of 387.16. It elutes relatively early from the C18 column, between chlorogenic and caffeic acids, even though it has a fairly large molar mass, and thus it must be a polar compound. In the cyano column, compound 7 also has a similar retention to chlorogenic acid (mw 354), which has five hydroxyl groups in addition to the acidic group. Thus, the unknown compound 7 probably has approximately three or four hydroxy groups. Since it has a good detector response in MS detection, it most probably is an acidic compound. According to the accurate mass obtained by TOF-MS, the molecular formula is $C_{21}H_{24}O_7$ or $C_{18}H_{28}O_9$. Further characterization with gas chromatography-MS of the silylated extract confirmed that the compound had the fragmentation pattern of a phenolic acid, and both fragmentation and the retention time suggested that there was, in addition to the COO^- group, most likely two additional hydroxy groups in the molecule.

19.9 CONCLUSIONS AND FUTURE PERSPECTIVES

In the analysis of phenolic compounds from plant-derived samples, several novel methods and techniques have been developed. The importance of proper sample pretreatment must be stressed, as the sample matrices are complex, and many of the target analytes are relatively labile. If possible, fresh samples should be analyzed. Novel extraction techniques utilizing, for example, PLE, SFE,

MAE, MSPD, and SAE, have proved to be useful in the extraction of phenolics from a wide variety of samples. Also, on-line combination of extraction is a very useful option in the analysis of reactive and labile analytes.

In the LC analysis of phenols, phenolic acids, and tannins, UPLC and fast-LC methods provide faster and more efficient separation of target analytes, in comparison with conventional HPLC analyses. Good separation can be obtained in 15 min or even shorter analysis times. Recently, several LC × LC systems have been developed for the analysis of phenolics as well. The benefit of UPLC and fast-HPLC methods is that the analysis time can be decreased without sacrificing the separation efficiency. LC × LC is a more complex system but is a good option for detailed characterization of plant samples, and it is also a very useful tool in the identification of unknown compounds, when it is connected to MS detection. MS detection, in combination with LC retention data and UV absorbance data, is crucial for reliable identification of phenolics in these complex samples. The use of TOF-MS for accurate molar mass determination and MS/MS modes for obtaining fragmentation data is increasing rapidly as the price and ruggedness of MS detectors are improving.

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20 Application of HPLC in Coumarin Analyses

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CONTENTS

Up til now, many different analytical methods have been published related to determination of wide variety of compounds in natural sources. One of them, high performance liquid chromatography (HPLC), considered one of the most versatile and adequately effective separation techniques, is frequently used in qualitative and quantitative determinations of natural compounds. HPLC has a number of advantages in comparison to other chromatographic methods, mainly because of its modular design, high-throughput of separation, and optional automated operation. It is now the most widely used technique for analyses of coumarins. Most coumarin-specific applications of HPLC include separations of crude extracts from selected higher plants as well as the analysis of complex coumarins besides their purification process.

Recently, liquid chromatography–mass spectrometry (LC-MS) and liquid chromatography–tandem mass spectrometry (LC-MS/MS) have proved to be some of the best alternative techniques for separation, identification, and quantification of coumarin compounds. However, those hyphenated techniques have one obvious limitation—the high cost, which explains the relatively small number of studies in that field.

Principally, the purpose of this chapter is to present information about HPLC methods and their recent applications in coumarin analyses. Therefore, in this chapter, brief introduction is given about applications of chromatographic methods, especially HPLC analyses, which are methodically different when featuring coumarins as the analytes. In addition, combined use of HPLC and various mathematical algorithms will be presented.

20.1 GENERAL OVERVIEW OF THE COUMARIN COMPOUNDS

Similarly to many of the other secondary metabolites stemming from natural sources, coumarins are attractive compounds with respect to their chemical properties, structures, biosynthetic pathways,

and biological activities. Historically, coumarin was first isolated by Vogel in 1820 from tonka beans (from the tonka tree), which belong to the Leguminosae family and were named *Dipteryx odorata* L. (*Coumarouna odorata* Aubl.) with pleasant odor. Actually, the earliest one was the daphnin (8-hydroxy-7-β-D-glucosyloxycoumarin) isolated from *Daphne alpina* (Thymelaeaceae) by Vauquelin in 1812, which is a glycoside-type coumarin, but the structure was not elucidated at that time [1,2].

Coumarins have a limited presence even in the plant kingdom, where they are considered most abundant. They are widespread in the angiosperms but rather rare in gymnosperms and lower plants. The majority of naturally occurring coumarins have been found in hundreds of higher plants from Dicotyledonae families, especially Umbelliferae and Rutaceae (distributed in almost all parts of the plants). Besides the Umbelliferae and Rutaceae, they are also present in the families Leguminosae and Compositae as well as in Rosaceae, Rubiaceae, and Solanaceae in free or glycosidic forms, and less commonly in other families such as Araliaceae, Caryophyllaceae, Euphorbiaceae, Hippocastaneceae, Myrtaceae, Oleaceae, and Thymelaeaceae. There are a few reports of coumarins being present in microorganisms, animals, fungi, molds, and mushrooms, but they have never been found in algae or mosses. Some coumarins in the liverworts *Plagiochasma tenue* Steph. and *Anthoceros laevis* L. have been tentatively identified as umbelliferone and scopoletin [2,3–5], and some isocoumarins have been found in *Wettsteinia inverse* [6].

Regarding their physiological role, the importance of coumarins is at this point uncertain. It is known that they have an important role in plant growth regulation, absorbing UV light and thus protecting plants both against over-irradiation and viral infections [7]. Both coumarin and coumarin derivatives are known to be formed as phytoalexins in response to plant diseases, injuries, or viruses. Ayapin and scopoletin are synthesized by the tuber of *Helianthus tuberosus* as a reaction to wounding or treatment with chemicals, so they are described as phytoalexins too [3].

The basic structure of coumarin (Figure 20.1) is a precursor for many different types of coumarins, and thus its physical properties should be mentioned. The molecular weight of coumarin is 146.15 Da, and the molecular formula is $C_9H_6O_2$. It forms colorless crystals in the shape of glasslike plates with a pleasant and characteristic odor; melting and boiling points are 68°C–70°C and 303°C, respectively, at atmospheric pressure. Coumarin dissolved in chloroform has a UV absorption maximum at 272 nm. It is entirely soluble in ethanol, chloroform, and oils but only sparingly soluble in both boiling and cold (20°C) water [1]. By the way, it is known that many of the coumarins are solid and normally crystalline in structure besides the greasy form and show well-defined melting points [2,8].

Coumarins are part of the group of benzopyrones called benzo-α-pyrones, which consist of a benzene ring joined to a six-member heterocyclic pyrone ring with an oxygen atom in α-position [9]; (Figure 20.1). They can be classified into the group of naturally occurring cinnamic acid lactones alternatively termed phenylpropanoids [2]. However, they are also referred to as 2H-1-benzopyran-2-on, 1,2-benzopyrone, *cis-o*-coumarinic acid lactone, coumarinic anhydride, *o*-hydroxycinnamic acid-8-lactone, and 2-oxo-1-benzopyran [1].

The phenylpropanoids are most commonly formed via the shikimate–chorismate biosynthetic pathway. It is reported that the coumarins are constituted as shikimate derivatives by deamination and hydroxylation of the phenylalanine to *trans*-hydroxycinnamic acid. The double bond of this acid is rapidly converted to the *cis* form by light-catalyzed isomerization, which results in formation of a coumarin [4,8].

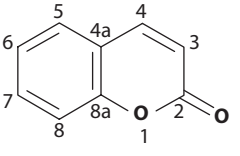

FIGURE 20.1 Coumarin (benzo-α-pyrone).

In the majority of coumarins, oxygenation at the C-7 position results in coumaric acid. The structure could also easily be hydroxylated at *ortho*- positions, thus constituting photocatalyzed isomers, or they can form lactones from *para*-hydroxylation of cinnamic acid [4,8]. Umbelliferone (7-hydroxycoumarin) is a precursor for the other coumarins, which are hydroxylated, methylated, *O*-prenylated, and even glycosylated at various positions on the cycle. Notably, prenylation is an important step, because of its presence in the biosynthetic pathway of polycyclic coumarins like furocoumarins and pyranocoumarins [4,8].

Higher plants synthesize coumarins via the shikimate–chorismate biosynthetic pathway responsible for the formation of the aromatic amino acids. In contrast, microbial coumarins are much more uncommon, although one important example is the biosynthesis of fungal aflatoxins: The coumarin core was found to be entirely derived from acetate via polyketide intermediates [2].

All these biosynthetic variations impart to coumarins countless natural forms. In the reports, there are different classifications of coumarins according to their structural types. Therefore, natural coumarins could be examined from different points of view [2,7,9]:

1. Simple coumarins: coumarin and its hydroxylated, alkoxylated, and alkylated derivatives of the benzene ring and their glycosides
2. Furocoumarins (dihydrofurocoumarins)
   a. Linear-type furocoumarins (psoralen type)
   b. Angular-type furocoumarins (angelicin type)
3. Pyranocoumarins (dihydropyranocoumarins)
   a. Linear-type pyranocoumarins (xanthyletin type)
   b. Angular-type pyranocoumarins (seselin type)
   c. Alloxanthyletin-type coumarins
4. Coumarins substituted in the pyrone ring, such as 4-hydroxycoumarins, 3-phenylcoumarins
5. Benzocoumarins (ellagic acid)
6. Coumestans (coumestrol, etc.)
7. More complex structures including the coumarin system (novobiocin, aflatoxin, etc.)
8. Isocoumarins

Up to now, coumarins' occurrence in natural sources and their comprehensive description have been described in many scientific studies. In general, fresh and dried plant materials are extracted with solvents of increasing polarity. It is well known that nonpolar coumarins are highly soluble in *n*-hexane and petroleum ether, while medium polar coumarins are soluble in chloroform, ether, ethyl acetate, and acetone; it is even possible to extract glycosides of coumarins using these solvents [2]. Mostly in organic solvents, it is possible to observe coumarins as either colorless or light yellow crystalline substances. Crystallization of the coumarins can be easily surveyed, either directly in the concentrated extract kept at laboratory conditions, during Soxhlet extraction, or during concentration and cooling steps [2,7–10].

Silica is frequently used as a stationary phase for segregating coumarins in crude extracts or fractions. Using combinations of *n*-hexane, ether, or ethyl acetate as mobile phases yields a satisfactory result. Adsorption chromatography is one of the most useful methods for the isolation of coumarins as well as other types of natural compounds. But degradation and partial losses of coumarins are a difficult problem during column chromatography in a basic or acidic environment. Another problem of coumarins during the separation process is that many of them have similar solubility in organic solvents; the sophisticated way out can be multiple recrystallization processes [2,7–10].

Studies focusing on the spectroscopic attributes of coumarin derivatives have been reported many times in the literature. UV absorption spectra are very useful for the determination of coumarins because of their distinctive chromophore groups conveniently visible under UV light.

Preparative thin-layer chromatography (TLC), preparative and semipreparative HPLC, and rechromatography or crystallization techniques have been effectively used for further separation and purification of coumarins. Coumarins are also detectable on gas chromatography (GC), and they can even be isolated at a preparative scale there [2,7–10]. Lozhkin and Sakanyan (2006) have also mentioned alternative methods, such as titrimetric, colorimetric, polarographic, and fluorescence analyses of coumarins [7].

Another important stage is the determination of the coumarins' structure. Spectroscopic analyses are very important in natural product chemistry not only for elucidating structures but also for impurity control. Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are very useful methods for confirmation of the expected variants of the coumarins. Occasionally, spectrofluorimetry and luminescence have also been used for this purpose [9]. Coumarin itself shows specific absorption bands at 274 and 311 nm in the UV absorption spectrum, which are attributed to the benzene and pyrone rings, respectively. The methyl substitutions at C-5, C-7, or C-8 lead to a bathochromic shift of the 274-nm peak but not of the 311-nm peak [2].

Mostly, natural coumarins have an oxygen substituent at the C-7 position. The spectra of 7-oxygenated coumarins (like umbelliferone) show strong absorption bands at ~217 and 315–330 nm with peaks, or shoulders at 240 and 255 nm. The UV spectra of 6- and 8-alkyl-substituted 7-oxygenated coumarins and of the linear and angular dihydrofurano- and dihydropyranocoumarins are all very similar. Anomalin (an angular-type pyranocoumarin) and deltoin (a linear-type furocoumarin) also have similar UV spectra [11], shown in Figure 20.2. The 5,7- and 7,8-dioxygenated coumarins also show spectra resembling those of the 7-oxygenated coumarins. Linear furocoumarins (psoralens) show four zones of absorption at 205–225, 240–255, 260–270, and 298–316 nm and thus could be easily distinguished from angular furocoumarins (angelicins) featuring maxima at 242–245 and 260–270 nm [2].

Infrared (IR) spectroscopy is used, especially for detecting functional groups and confirming the identities of unknown compounds. Moreover, conjugated lactone conformation can easily be revealed in the spectra. The carbonyl stretching (–C = O) frequency in coumarins (α-pyrones) is observed in the region 1750–1700 cm^{-1}, whereas in chromones (γ-pyrones) it is found at ~1650 cm^{-1}. The –C = C– groups of the aromatic ring are in the region of 1620–1470 cm^{-1} [2,7].

Inevitably, the most important spectroscopic method for structural elucidation in natural product chemistry is NMR. Observation of a pair of doublets located at δ 6.1–6.4 ppm and 7.5–8.3 ppm (*J* 9.5 Hz) and belonging to H-3 and H-4, respectively, in the ^{1}H-NMR spectrum (in $CHCl_3$) of a

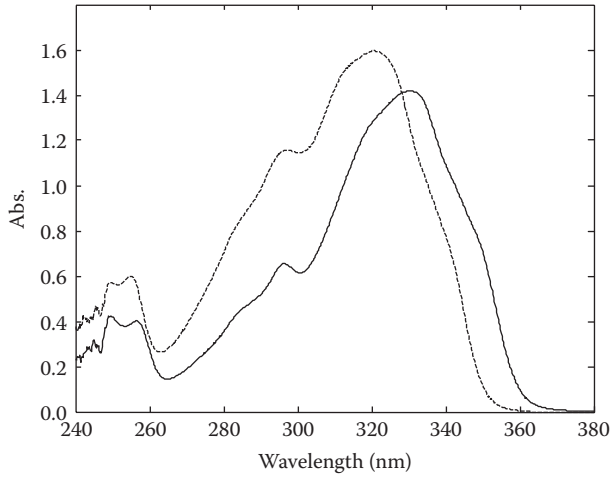

FIGURE 20.2 Absorption spectra of anomalin (----) and deltoin (—) in the mixture of *n*-hexane and ethyl acetate (50:50, v/v). (Reprinted from Tosun, A., Bahadır, Ö., and Dinç, E., *Chromatographia*, 66, 677–683, 2007. With permission from Bontelme, Vieweg-Publishing, Germany.)

natural product strongly indicates a coumarin unsubstituted at the pyrone cycle. The majority of natural coumarins have an oxygen atom at the C-7 position that leads to an increase in the electron density at the C-3 position compared to coumarin, so H-3 results in resonance in a higher field. The signal from H-5 is found at δ ~7.3 ppm, downfield from the H-6 resonance at δ ~6.8 ppm (*J* 9 Hz) [2].

The position of the aromatic proton in 5- and 8-alkoxypsoralens (in furocoumarins) can be easily defined by the ^{1}H-NMR spectrum. In the 8-alkoxy series, the H-4 and H-2′ signals overlap, and H-5 appears at δ 7.3–7.4 ppm, whereas for 5-alkoxypsoralens H-4 is shifted downfield, H-2′ upfield, and H-8 resonates at δ 7.0–7.2 ppm. In addition, aromatic methoxy groups generally resonate in the range δ 3.8–4.4 ppm, and aromatic methyl groups at δ 2.45–2.75 ppm. However, if the methoxyl group is attached to the benzene ring, protons appear at about 3.8 ppm [2].

By the way, the presence of an unsubstituted furan ring is easily recognizable from the pair of doublets, *J* ~2.5 Hz, which arise from H-2′ and H-3′. The signal of H-2′ resonates at δ ~7.5–7.7 ppm, while the latter are found at δ ~6.7 ppm in the linear series and at δ ~7.0 ppm in the angular series [12]. Natural pyranocoumarins are characterized by *geminal* methyl groups that resonate as a six-proton singlet at ~δ 1.45 ppm. Two pairs of doublets are centered at δ 5.3–5.8 ppm (H-3′) and at 6.3–6.9 ppm (H-4′), *J* = 10 Hz [2].

The identity of acid moiety of the ester parts in coumarin structure such as acetic acid, angelic acid, senecioic acid, 2-methylbutyric acid, isovaleric acid, and tiglic acid can usually be determined from ^{1}H-NMR spectra. A sharp singlet of methyl group in acetates is the most specific signal at δ 2.0–2.2 ppm [2,13–16]. Measurements of nuclear overhauser effects (NOE) have proved helpful in determining the geometry of certain unsaturated side chains and have been especially useful in the structural elucidation of a number of coumarins having substituents [2].

In addition, ^{13}C-NMR spectroscopy is also a convenient method in the structural elucidation of natural products by the development of Fourier-transform methods and computers. Natural coumarins have been examined by the assignment of ^{13}C chemical shifts and carbon–proton couplings. The chemical shift of the carbonyl–carbon atom has been found to be approximately the same (~160 ppm) in most of the coumarins. The effect of hydroxy- and methoxy-groups present on the benzene ring is quite characteristic because of the newly formed quaternary carbon atom found at approximately 30 ppm downfield [2]. Nonetheless, the extensive analysis of ^{13}C-^{1}H long-range couplings has been reported by researchers in the literature many times. Basically, the chemical shifts in the coumarin skeleton itself (in parts per million [ppm] relative to tetramethylsilane (TMS) in $CHCl_3$) could be given as follows: C-2: 160.4; C-3: 116.4; C-4: 143.6; C-4a: 118.8; C-5: 128.1; C-6: 124.4; C-7: 131.8; C-8: 116.4; and C-8a: 153.9 [2,17–19].

Considerable interest has been shown in the MS of natural products. Fragmentation patterns resulting from the electron-impact ionization of many natural coumarins have proved to assist greatly in structure elucidations. High-resolution fast atom bombardment–mass spectrometry (HRFAB-MS) in particular has been increasingly employed for the determination of molecular formulas by accurate measurement of the molecular ion weights. Coumarin gives a strong molecular ion (M^+, *m/z* 146, 76%) ionized by electron impact (EI) and a base peak (*m/z* 118, 100 %) that is formed directly from the molecular ion by loss of carbon monoxide (–CO), a highly stable natural particle, from the pyrone ring (Figure 20.3). This fragmentation has been supported by accurate mass measurement of the formed fragment ion [2].

Mass fragmentation is very useful for the structural elucidation, especially for the side chains that are esters. Two competing fragmentations of the five-carbon side chain are observed in the mass spectra of *C*-prenylated methoxycoumarins. For example, suberosin (Figure 20.4) has been shown to lose a methyl radical from the prenyl group and give rise to highly conjugated ion at *m/z* 229 as a base peak [2].

A very specific fragmentation process is observed both for simple coumarins and for furocoumarins with a furan ring. For example, in methoxyfuranocoumarins such as xanthotoxin (Figure 20.5), where loss of a methyl radical can give rise to a conjugated oxonium ion, this process predominates [2].

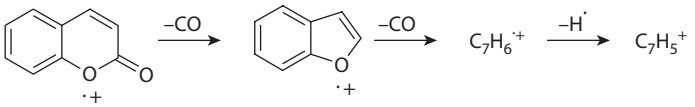

FIGURE 20.3 Fragmentation pattern of coumarin. (From Murray, R.D.H., Méndez, J., and Brown, S.A., *The Natural Coumarins, Occurrence, Chemistry and Biochemistry*, John Wiley & Sons, Chichester, 1982. With permission.)

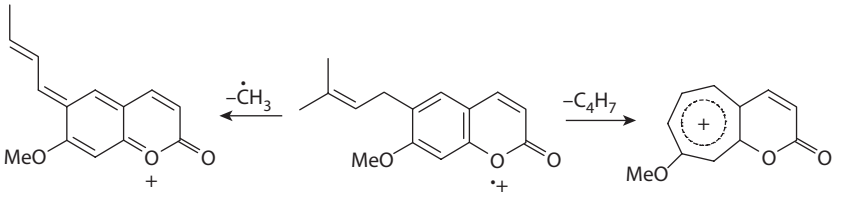

FIGURE 20.4 Fragmentation pattern of suberosin. (From Murray, R.D.H., Méndez, J., and Brown, S.A., *The Natural Coumarins, Occurrence, Chemistry and Biochemistry*, John Wiley & Sons, Chichester, 1982. With permission.)

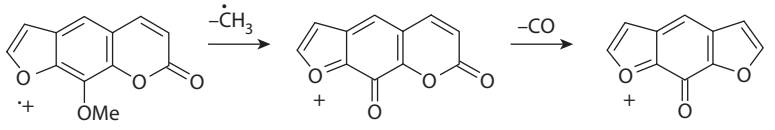

FIGURE 20.5 Fragmentation pattern of xanthotoxin. (From Murray, R.D.H., Méndez, J., and Brown, S.A., *The Natural Coumarins, Occurrence, Chemistry and Biochemistry*, John Wiley & Sons, Chichester, 1982. With permission.)

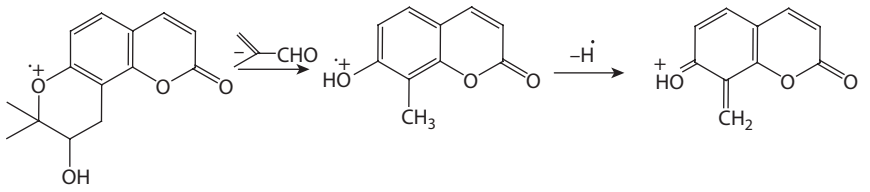

FIGURE 20.6 Fragmentation pattern of lomatin. (From Murray, R.D.H., Méndez, J., and Brown, S.A., *The Natural Coumarins, Occurrence, Chemistry and Biochemistry*, John Wiley & Sons, Chichester, 1982. With permission.)

In pyranocoumarins, e.g., the dihydropyran loose both methyl groups (Figure 20.6) as being in lomatin (it is isomer with columbianetin) [2].

The 3′,4′-diaclyoxydihydropyranocoumarins also fragment mainly by a pathway leading to the stable benzopyrylium ion; the 3′-acyloxy group is split out preferentially as carboxylic acid and can be distinguished from the 4′-acyloxy group in both the angular and the linear series of diesters (Figure 20.7). This is very important information when we determine the ester group position in 3′,4′-dihydopyranocoumarins [2].

In a recent study, the stereochemistry of a new isolated pyranocoumarin from *Seseli gummiferum* ssp. *corymbosum* was discussed with respect to its fragmentation pattern as well as its chemical behavior [13].

Besides the hard-ionization MS techniques, predominantly EI-MS, soft-ionization MS methods such as field ionization (FI), chemical ionization (CI), field desorption (FD), and electrospray ionization (ESI) can give complementary information to that available from conventional high-resolution and low-resolution electron-impact studies.

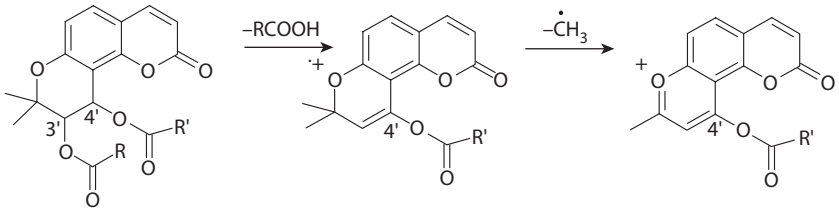

FIGURE 20.7 Fragmentation pattern of 3′, 4′-diaclyoxydihydopyranocoumarins. (From Murray, R.D.H., Méndez, J., and Brown, S.A., *The Natural Coumarins, Occurrence, Chemistry and Biochemistry*, John Wiley & Sons, Chichester, 1982. With permission.)

Coumarins are stereospecific compounds consisting of an asymmetric carbon atom in the furan cycle of a substituted dihydroangelicin and the pyran cycle of substituted khellacton. The stereochemistry of the double bonds in coumarins was proved by spectroscopic evidence and confirmed by a specific synthesis of each compound. The unsaturated five-carbon units and unsaturated acyl groups indicate the stereochemistry in coumarin structure, and they were identified in the literature. The absolute configurations of the majority of linear and angular dihydrofuranocoumarins containing one chiral center (at C-2′) have been firmly established by researchers. Shibata and Okuyama also debated the structural elucidation and stereochemistry of seselin- and xanthyletin-type pyranocoumarins, and psoralen- and angelicin-type furocoumarins in their study [20]. Nodakenetin and marmesin can be mentioned as an interesting example of enantiomeric forms of the same coumarin that are found in different sources [21].

Dihydropyranocoumarins have two chiral centers at the C-3′ and C-4′ positions, which have the diesters (+)-*cis*-khellactone and its enantiomer (–)-*cis*-khellactone in absolute stereochemistry. It is also known that ^{1}H-NMR spectra are useful for determination of relative configurations in the khellactones and their diesters. For *cis*-khellactone derivatives, $J_{3',4'}$ 5.0 Hz is consistently observed with little difference in the chemical shift of the *geminal* methyl groups at C-2′, which appear as a slightly broadened singlet. By the way, a variation in $J_{3',4'}$ of 3.0–6.9 Hz is observed for *trans*-khellactone derivatives. This is accompanied by a difference ranging from 0.08 to 0.20 ppm for the chemical shift of the *geminal* methyl signals, which therefore appear as a discrete singlet. The $J_{3',4'}$ in *cis*-diol and diesters of dihydropyranocoumarins is invariably 4 Hz regardless of the substituent; it is 5 Hz in the *cis*-khellactone series. *Trans*-diesters in the linear series consistently show $J_{3',4'}$ 6 Hz, again with well-separated *geminal* dimethyl singlets, whereas *cis*-diesters show $J_{3',4'}$ 4 Hz and a singlet signal at δ 1.45 for the *geminal* methyl groups, which are rather close to each other [2,22,23].

The coumarins were first synthesized chemically by W.H. Perkin in 1858 as a vegetable perfume; the classical reaction for the formation of coumarin is called the Perkin reaction and is comprised of heating *o*-hydroxybenzaldehyde with sodium acetate and acetic anhydride at 180°C. The most important step is the formation of pyrone ring Figure 20.8 [2]; after that, there are several methods to prepare the substituted coumarins so as to link functional groups into this main structure [1,2,9].

These chemical investigations are used mainly to confirm structural features predicted from elemental and spectroscopic analyses and to distinguish between isomeric structures. The structures of the coumarins can be confirmed either by Perkin condensation or by the Pechmann or Knoevenagel reaction and some other techniques [7]. The characteristic reaction of the coumarins is splitting or fragmentation of the lactone ring (Figure 20.9) in alkaline solutions then slow hydrolysis yielding coumarinates (a yellow solution) [2,9].

Acidification leads to relactonization and regeneration of the native coumarin structure. However, in many cases the original coumarin is not recovered. For coumarins having a free phenolic group at the C-5 position, the result is formation of an isomer of the original coumarin. 7-methoxycoumarin is much harder to hydrolyze than coumarin. Prolonged treatment of coumarin with hot dilute

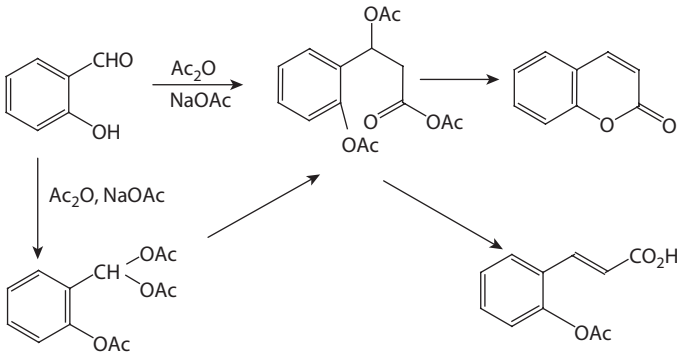

FIGURE 20.8 Perkin reaction. (From Murray, R.D.H., Méndez, J., and Brown, S.A., *The Natural Coumarins, Occurrence, Chemistry and Biochemistry*, John Wiley & Sons, Chichester, 1982. With permission.)

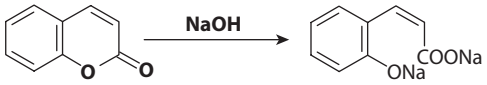

FIGURE 20.9 Formation of coumarinate salt in alkaline conditions.

aqueous sodium hydroxide results in *cis* to *trans* inversion, with acidification giving *ortho*-hydroxy-*trans*-cinnamic acid (*o*-coumaric acid) in a way that is not absolutely clarified [2,9].

More vigorous treatment of coumarins with alkali (for example, at first with hot 15% potassium hydroxide (KOH) yields acetic acid together with phenolic acids or ketones derived from a split of the lactone ring. Many angular and linear 3′, 4′ dihydropyranocoumarins are known to feature a frequently encountered *cis* relationship of the two acyloxy groups. Most of the natural diesters originating from an angular type are now known to have the same absolute stereochemistry as the parent diol, which is known by the trivial name (+)-*cis*-khellactone. Mild hydrolysis of its diesters, treated with 5% aqueous potassium hydroxide in dioxan overnight at laboratory conditions, gives, for example, a mixture of (+)-*cis*-khellactone and (–)-*trans*-khellactone (Figure 20.10) in approximately equal amounts [2,9].

Many of the coumarins have prenyl, geranyl, or farnesyl ether residues. Such moieties, and also terminally functionalized geranyl or farnesyl residues in which the allyl ether system is still present, characteristically undergo simple hydrolytic cleavage under mild acidic conditions. When we use acetic acid (containing concentrated sulfuric acid) at 100°C, the reaction results in predecessors of the coumarins that have alkyl substituents as a separate alkyl group [2].

It is apparent that coumarins are sensitive to alkaline and acidic conditions. Another serious problem with alkali is the hydrolysis of ester linkages. Esters of phenolic coumarins are extremely labile, and natural examples have not yet been reported. Heating natural coumarin prenyl ethers, even in a high vacuum, should be avoided on account of their ability to undergo the Claisen allyl ether rearrangement [2].

It is known that the major detoxification mechanism of the coumarins in animals involves hydroxylation of the benzene ring followed by a conjugation reaction. The metabolites were identified in studies carried out on microbes (*Arthrobacter*, *Aspergillus niger*). In higher plants, coumarin is also metabolized to some extent [2].

20.2 ANALYTICAL METHODS USED FOR DETERMINATION OF COUMARINS

Coumarins are found in many important plants used as foodstuffs, such as spices, vegetables, and fruits, together with their usage as ornaments, and thus we should not underestimate their identification and profound biological importance. Because of these reasons, a wide range of analytical techniques exist for the qualitative and quantitative determination of coumarins, including traditional methods and general techniques [9,24].

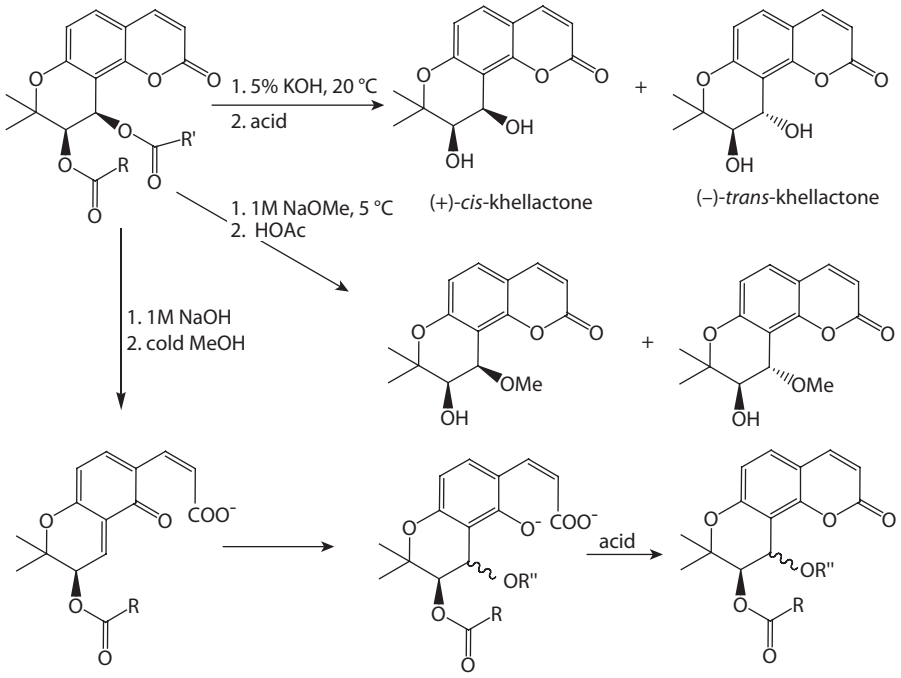

FIGURE 20.10 Chemical treatment of angular-type 3′,4′-dihydropyranocoumarins. (From Murray, R.D.H., Méndez, J., and Brown, S.A., *The Natural Coumarins, Occurrence, Chemistry and Biochemistry*, John Wiley & Sons, Chichester, 1982. With permission.)

TLC is a powerful method for separation and identification of natural compounds because of its relatively easy execution and quick response [25]. It is based on the adsorption principle of chromatography with a coated stationary phase and an evaporating liquid mobile phase. It is used mostly in qualitative analysis, but it is possible to use it for quantitative analysis of coumarins. A wide variety of literature can be found concerning TLC of coumarins using well-selected solvent systems [7,10].

The most important physical property of natural coumarins eluted on the TLC plate is the fluorescence in response to UV light at 365 nm. This characteristic feature enables us to identify and recover compounds without using chromogenic reagents. Based on the wavelength absorption, it is often possible to predict a classification into one of the coumarin categories. Furocoumarins can readily absorb light and emit blue or yellow fluorescence under long-wavelength UV light (UVA, 320–380 nm). Meanwhile, simple coumarins emit bright blue fluorescence; pyranocoumarins have distinctive purple fluorescence in reaction to UV irradiation of the chromatographic plate.

After developing the chromatogram, coumarins can be visualized by treating them with alkali such as 5% or 10% potassium hydroxide or ammonium solutions. Intensification of the fluorescence in the presence of alkali is indicative of phenolic groups in the coumarin. The fluorescence intensity of a coumarinic salt can be exploited for determination of the coumarin concentration [2].

There are some chromogenic reactions that can be utilized for the purpose of characterization of the coumarins; one in particular, the "Emerson reagent," is very useful for distinguishing between linear (psoralens) and angular (angelicins) furocoumarins. Moreover, we can exploit a very useful "brightness and color modulation" by treating the chromatographic plate with methanol:sulfuric acid (1:1) as well as potassium hydroxide and ammonium solutions, which can help with interpretation of the spots on TLC plates [2,9,13].

Since the 1960s and 1970s, with advances in HPLC and GC, coumarin analysis has been becoming easier and easier to perform utilizing these two rapidly evolving sophisticated techniques [9]. The underlying principle of their operation is very complex but could be explained basically as the

following: HPLC and GC separation is based on the number and intensity of analytes' interactions with the stationary and mobile phase.

In some samples (for example, the common additive vanilla extract), coumarins could be determined using a simple mixture of methanol:water as the mobile phase (40:60 v/v, with addition of 0.1% acetic acid) and a C_{18} column equipped with a UV detector, and this method could be used to assess quality. In another example, an ethanolic extract was eluted using a C_8 column by gradient elution with water:phosphoric acid and methanol (at pH 3.0 and detected at 210 nm) in order to determine the hydroxycoumarins in the bark of *Fraxinus ornus*. The determination of coumarins in cinnamon and cassia was carried out on a LiChrosorb RP-8 reversed-phase column eluted with water:methanol:acetonitrile:tetrahydrofuran (60:12:20:8, v/v/v/v) using vanillin as an internal standard [9].

The compounds found in complex mixtures from plant extracts are usually difficult to separate in a single chromatographic run. For this reason, combined HPLC and TLC systems were applied to separate the coumarins in *Angelica officinalis*. The HPLC gradient was optimized on a RP-18 column using DryLab for Windows software, and TLC was performed on silica plates [26].

When drugs are hard to detect by routine toxicological analysis, HPLC-MS, HPLC-MS/MS, or capillary electrophoresis linked to MS and MS/MS provide the additional resolution required for otherwise undetectable compounds. Many drugs, including coumarin-based anticoagulants, were investigated easily and rapidly using these chromatographic systems [27].

GC is very convenient method for the separation of thermally stable and volatile organic and inorganic compounds. Coumarins can be analyzed as corresponding acetates using GC even in combination with MS [2]. Basically, separations of coumarins are carried out on a fused-silica capillary with helium as the carrier gas; the temperature increments for the different gradient methods vary from 8°C/min or 10°C/min up to a final temperature of 300°C. Generally, coumarin analyses on GC are preferred due to their easy derivatization [9]. Vegetable juices containing umbelliferone, psoralen, and bergapten were analyzed with great success by GC-MS [9]. Ayapin and scopoletin purified from *Helianthus tuberosus* L. were analyzed by GC-MS (EI ionization, 70 eV) on a DB5 column (30 m × 0.32 mm) [3].

Moreover, 5,7-dimethoxycoumarin together with other polar molecules from the leaves of *Carica papaya* were successfully analyzed by GC-MS (EI ionization, 70 eV) equipped with an SPB-5 capillary column (length: 30 m; ID: 250 μm; film thickness: 1.4 μm). Helium was the carrier gas; the inlet temperature was 280°C, while the oven temperature was initially 100°C (held for 3 min) and then was increased to 315°C at a rate of 20°C/min. Detection was performed in selected ion monitoring mode, and peaks were identified and quantified using target ions [28].

Headspace solid-phase microextraction (HS-SPME-GC-MS) and LC-ESI-MS methods were also successfully utilized for the determination of coumarin, vanillin, and ethyl vanillin in vanilla products [29]. There have also been studies on rather peculiar methods scarcely used in natural product chemistry. Polarography of the coumarins was accomplished by Smyth et al. [24]. Capillary electrophoresis is also not frequently used for the analysis of coumarins. So there are only a few reports, one of which involved the separation of 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide, and warfarin. The separations of the compounds were carried out on an untreated silica capillary and detected at either 210 nm or 320 nm, using a phosphate buffer-based solution as an electrolyte [9].

Preparative high-speed countercurrent chromatography (HSCCC) was also used for isolation and purification of coumarins from the Chinese traditional medicinal herb *Notopterygium forbessi* Boiss. with a pair of two-phase solvent systems (light petroleum:ethyl acetate:methanol:water, 5:5:4.8:5 and 5:5:4:5) [30]. Moreover, it was also used for isolation and purification of coumarins such as imperatorin, isoimperatorin, and oxypeucedanine from *Angelica dahurica* (Fisch. ex Hoff.) Benth. et Hook. f [31]. In addition, HSCCC was used by the same research group to perform separation and purification of coumarins from *Cnidium monnieri* (L.) Cusson [32] as well as from *Cortex Fraxini* (a Chinese herbal drug) and *Peucedanum praeruptorum* [33,34].

20.3 BIOLOGICAL ACTIVITIES OF COUMARINS

The various biological and pharmacological activities of coumarins, have been known for a long time. They cause a diversified spectrum of effects, for example, inhibition/induction of enzymes, modulation of immune cells, viral inhibition, antioxidant and antineoplastic activities, and, last but not least, endocrine-system stimulation. Many coumarin compounds influence our life span through foodstuffs, and understanding their biological activities should be on the same level as identifying their chemical structures.

Coumarin itself is an odoriferous compound, thus widely used in the perfume industry [7]. Many of the umbelliferous plants such as *Apium graveolens* (celery), *Petroselinum sativum* (parsley), *Pastinaca sativa* (parsnip), and *Angelica archangelica* (angelica) are used for their culinary significance, but they contain furocoumarins causing dangerous phototoxicity [4,8,9]. As mentioned in one of the preceding sections, some of them, namely, phytoalexins, exhibit potent antimicrobial and antifungal activities and are able to eradicate *Candida albicans* in vitro [4,8,9]. Recently, a coumarin called pavietin was also confirmed to be a fungicidal compound [35].

Moreover, some simple coumarins that are substituted with esters or carboxylic acid were found to exhibit potent inhibitory activities against both gram-positive and gram-negative bacteria. The presence of a phenolic hydroxyl group and/or carboxylic acid was found to be necessary for higher antimicrobial activity against *Helicobacter pylori* [36].

If it is administered perorally, coumarin exhibits hepatotoxic activity [37]. Because of this side effect, it was already banned by the Food and Drug Administration in the 1950s, being classified as a category 1 carcinogen and hepatotoxin, based on animal data [1]. Coumarin itself was marketed in France until late 1996. French government regulations, considering the 1988 European directive, specify that residual coumarin content in food and beverages must not exceed 2 mg/kg [8].

Coumarin exhibits cytotoxic activities against a number of human tumor cells but unfortunately against normal cells as well. It has been used in treatment and prophylaxis of malignancies in melanoma and renal tumors. It demonstrates activity against tumor cells directly or indirectly via modulation of the immune system response, thus protecting the host against occurrence and recurrence of tumor. Obviously, one can find numerous studies in which coumarins affected growth of tumor cells both in vitro and in vivo [8,9].

Coumarin and its derivative 7-hydroxycoumarin inflict cytostatic effects on a number of human malignant tumors, especially renal carcinoma, colon carcinoma, breast carcinoma, glioblastoma, prostatic carcinoma, and lymphoblastoma. It was discovered that coumarin and its 7-hydroxy derivative inhibit the growth of a rat mammary adenocarcinoma cell line and a human bladder carcinoma cell line in vitro as well [8,9].

Isolated compounds from *Angelica dahurica* (Apiaceae) were examined regarding their cytotoxic activity against L1210, HL-60, K562, and B16F10 tumor cell lines using the MTT cell assay. It was discovered that pangelin and oxypeucedanin hydrate acetonide exhibited the most potent cytotoxic activity against all selected tumor cell lines, with IC_{50} values ranging between 8.6 and 14.6 μg/mL [4,8,38].

The linear-type pyranocoumarin xanthyletin is found to be moderately active at inhibiting nucleic acid synthesis in *Ehrlich ascites* tumor cells. Chartreusin formed by *Streptomyces chartreusis* as a complex coumarinic structure damages leukemia cells [2], as does one of the linear-type pyranocoumarins called clausarin, which was isolated from *Citrus sinensis* (Rutaceae) [39].

It should be noted that a diet supplemented with the *Citrus* coumarin auraptene (7-geranyloxycoumarin), structurally close to umbelliprenin, significantly reduced growth and number of metastatic lung tumors in mice bearing B16BL6 murine melanoma. It also reduces 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced free radical generation besides nitric oxide synthase and cyclooxygenase-2 expression in lipopolysaccharide-stimulated inflammatory cells and decreases lipid peroxidation and experimental carcinogenesis in rats. Umbelliprenin (7-farnesyloxycoumarin) isolated from *Ferula szowitsiana* also exhibited cytotoxic activity against metastatic malign melanomas [40]. Furocoumarins such as imperatorin and deltoin have been found to be potent inhibitors of nitric

oxide (NO) synthesis according to the inducible nitric oxide synthase (iNOS) inhibition bioassay. Their IC_{50} were 17.3 and 11.6 μg/mL, respectively [41]. This method introduces among others a new approach for cancer chemotherapy.

The coumarins 9-hydroxy-4-methoxypsoralen and alloisoimperatorin, isolated from *Angelica dahurica* Benth. et Hook. (Umbelliferae), were proved to be potent antioxidants in vitro [42].

The angular-type pyranocoumarins of *Angelica furcijuga* KITAGAWA such as hyuganin A–D, anomalin, pteryxin, isopteryxin, and suksdorfin inhibited lipopoly saccharide (LPS)-induced NO synthesis and/or TNF-α production in mouse peritoneal macrophages and isoepoxypteryxin inhibited D-GalN-induced cytotoxicity in cultured primary rat hepatocytes. Hyuganin A, anomalin, and isopteryxin caused a reduction in cell viability through the tumor necrosis factor (TNF)-α pathway in L929 cells [43]. Isoepoxypteryxin, an acetylated khellactone from methanolic root extract of *Angelica furcijuga* KITAGAWA, showed protective activity against D-GalN-induced cytotoxicity in primary cultured rat hepatocytes. It should be noticed that even the slightest structural deviations cause a dramatic change in the bioactive properties [44].

An angular-type furanocoumarin from *Angelica edulis* Miyabe, edulisin V, having one angeloyloxy group, showed potent inhibitory activity of 12-*O*-tetradecanoylphorbol 13-acetate (TPA)–stimulated ^{32}Pi incorporation into phospholipids in cultured HeLa cells [45]. The same effect was demonstrated by archangelisin, although it was more profound due to the presence of two angeloyloxy groups instead of one in 8(*S*),9(*R*)-9-angeloyloxy-8,9-dihydro oroselol [46].

Seselin (an angular-type pyranocoumarin), as well as xanthyletin and decursin (linear-type pyranocoumarins), should not be forgotten in the list of cytotoxic agents, in addition to some pyranocoumarins isolated from a chloroform extract of *Peucedanum japonicum*, such as (+)-*trans*-khellactone, (+)-*trans*-4′-acetyl-3′-tigloylkhellactone, and (+)-*cis*-4′-acetyl-3′-angeloyl khellactone [47–49]. Coumadin (sodium warfarin) is well-known anticoagulant also exhibiting antineoplastic effects. Thornes at al. showed that both coumarin and coumadin possess immunomodulatory effect in vivo on an animal model [8,9].

The induction of proteolysis is the next major attribute of coumarins. Coumarin and other benzopyrones increase the numbers of macrophages and their level of activity, thus decreasing protein-abundant edemas. So benzopyrones have been very effective in experimental models of acute and chronic inflammation, such as burns, cerebral vascular traumas, various forms of ascites, and adult respiratory distress syndrome [9,50].

Almost without exception, 4-hydroxycoumarins such as the coumarin antibiotics novobiocin and coumermycin are the most potent compounds with respect to their anticoagulative effect in blood. Dicoumarol is a well-known anticoagulant that causes a hemorrhagic disease in cattle grazing on *Melilotus* species, as was proved in Canada. Dicoumarol (a biscoumarin) arising from fungal contamination of *Melilotus officinalis* (L.) Pallas is being used as an anticoagulant in the United States. Nowadays, sodium warfarin (warfarin salts) and nicoumalone synthesized based on the dicoumarol structure are widely used as anticoagulants [2,4,8]. Warfarin is the best-known 4-hydroxycoumarin derivative; it is a vitamin K antagonist that is even used clinically in cases of venous thromboembolism, threatened stroke, and acute myocardial infarction because of its anticoagulative action, as well as being used as a rat poison [4,8,9,50].

Aflatoxins are hepatotoxins and carcinogenic agents occurring naturally as biosynthetic defense metabolites in various strains of *Aspergillus* during storage of plant materials [8,50].

It has long been known that various plant species inflict cutaneous hyperpigmentation. *Ammi visnaga* (Apiaceae) and *Psoralea* (Fabaceae) were used by the Egyptians as traditional ayurvedic medicine to treat vitiligo symptoms. These cutaneous effects reflect phototoxicity caused by linear furanocoumarins such as psoralen, bergapten, and xanthotoxin, in contrast to angular ones, which show only a weak activity. Furocoumarins are well known for their photosensitization activity because of the extended chromophore of these compounds. The furocoumarin nucleus is a prerequisite for the photosensitizing effect, which occurs especially in response to either sunlight or 365-nm UV radiation. These compounds may be the plants' natural protection against sunlight

over-irradiation [4]. The photosensitizing effects of linear furocoumarins are not confined to skin cells but also affect bacterial, fungal, and other types of mammalian cells; DNA viruses; and bacteriophages [2,4,8].

Preparations extracted from Apiaceae and Rutaceae plants containing psoralens have been used to promote skin pigmentation in the cases of vitiligo and psoriasis. Administered perorally combined with xanthotoxin and UVA radiation, these preparations are part of a therapy that is referred to as PUVA (psoralen + UVA) or its modification, photodynamic therapy, in which the drug is activated by irradiation with UV light at 320–400 nm [2,4,8].

Bergamot oil, which contains bergapten, is used as photodynamic sensitizer in sun lotions and cosmetics; it safely increases melanin production by promoting melanogenesis. Although bergamot oil finds its application in the cosmetics, pharmaceutical, and food industries, strong limitations have been imposed on its use due to photoactive impurities in the forms of other coumarins [2,4,8]. Meanwhile, Figoli et al. have mentioned an alternative technique termed pervaporation for obtaining a high-quality bergamot oil without bergapten [51].

Bergapten, xanthotoxin, isopimpinellin, and isoimperatorin isolated from *Tetradium daniellii* (Rutaceae) were investigated for their insect antifeedant activity. Xanthotoxin, bergapten, isopimpinellin, and bergamottin were found potentially effective against *Spodoptera littoralis* and *Heliothis virescens* [52]. The volatiles obtained from *Trifolium glanduliferum* and *T. strictum* were shown to deter the red-legged earth mite (*Halotydeus destructor*) from feeding. The coumarin, together with β-ionone found in the volatiles of *Trifolium glanduliferum* and *T. strictum*, showed strong deterrent activity at 100 ppm [53].

It has been known for a long time that coumarins have antioxidant activities depending on their hydroxyl groups [50,54]. When two hydroxyl groups are present, as in esculin isolated from the bark of *Aesculus hippocastanum* L. (common horse chestnut, Hippocastaneceae), it is a venous corroborant and has vascular protective effects [8]. Esculin was also determined to be present as a major compound in the bark of *Faxinus ornus*, an anti-inflammatory and vitamin P-like agent. Esqusan, Esflazid, and Anavenol are anti-inflammatory drugs based on esculin [8,9,55]. Esculetin, 4-methylesculetin, scopoletin, and 4-methyl-7-hydroxycoumarin were proved to be competitive inhibitors of xanthine oxidase (XO), and they are also potent superoxide-suppressing agents [56]. Esculetin and fraxetin showed potential anti-oxidative activity in many screening systems, higher than that of the corresponding glucosides fraxin and esculin and comparable to that of other well-known antioxidants [55,57,58].

Another group of coumarins, coumestans, are estrogenically active components known as 3-phenylcoumarins (3-phenylcoumarin, coumestrol). Khellin, an isocoumarin (furanochromone) from *Ammi Visnaga* Lam. (Apiaceae), exhibits a spasmolytic and vasodilating activity. It has been used in preventive therapy of angina pectoris in several European countries [2,4,8]. Visnadin, an angular-type pyranocoumarin known as a coronary vasodilator and positive inotropic, bradycardic, and spasmolytic agent, probably as a result of its calcium-blocking activity in vitro, has been extracted and marketed for its coronary vasodilating effect and is promoted as having a favorable action on senile cerebral insufficiency [2,4,8]. Campestrinol, grandivitin, and decursinol acetate, which are pyranocoumarins from the root of *Seseli grandivittatum*, have spasmolytic and coronarodilating activity [59].

Scoparon, a simple coumarin, is an anti-asthmatic coumarin derivative extracted from the traditional Chinese herb "Yin Chen." Scoparone also significantly relaxes tracheal smooth muscle and reduces total cholesterol and triglycerides amounts. Osthole also causes hypotension in vivo and inhibits platelet aggregation and smooth muscle contraction in vitro [50,60].

Nodakenin, a furocoumarin, was found to inhibit acetylcholinesterase activity in a dose-dependent manner ($IC_{50} = 84.7$ μM) in an in vitro study [61]. Recently, it has been reported that decursinol and decursin exhibited significant neuroprotective activity against glutamate-induced neurotoxicity in primary cultures of rat cortical cells and improved scopolamin-induced amnesia in vivo [47].

Suksdorfin isolated from fruit of *Lomatium suksdorfii* inhibited HIV-1 replication in H9 lymphocytes [62]. After that, many of the synthetic dihydroseselins, based on the structure of suksdorfin, were evaluated regarding their anti-HIV activity in vitro. Among them, 3′,4′-di-*O*-(–)-camphanoyl-(+)-*cis*-khellactone showed potent inhibitory activity and remarkable selectivity against HIV replication [63].

A simple coumarin, osthenol, from *Angelica koreana* Max. showed strong inhibitory effects on 5α-reductase type I in LNCaP cells (LNCaP cells are derived from an androgen-sensitive human prostate cancer cell line widely available for use in preclinical investigations) with an IC_{50} value of 0.1 μg/mL [64]. Moreover, the anti-proliferative effects of isopentenylated coumarins isolated from leaves of *Phellolophium madagascariense* Baker were examined on L1210 mouse leukemia and on human prostate cancer hormone-sensitive LNCaP and hormone-independent PC3 and DU145 cell lines [65].

20.4 HPLC IN ANALYSES OF COUMARINS

20.4.1 Normal-Phase HPLC in Coumarin Analyses

The use of normal stationary-phase columns for resolution of natural compounds is limited in the studies. Linear furocoumarins such as psoralen, bergapten, xanthotoxin, and isopimpinellin isolated from three varieties of *Apium graveolens* were examined by normal-phase HPLC equipped with a variable wavelength detector set at 250 nm; the mobile phase consisted of a mixture of ethyl acetate (0.1%) and formic acid (0.1%) in chloroform [66].

Recently, the normal-phase HPLC method was used to evaluate coumarins quantitatively. Osthol and corybocoumarin were isolated from aerial parts of *S. gummiferum* subsp. *corymbosum* as major compounds [13]. They were determined in three of the *Seseli* species collected from different localities in Turkey. In this study, the coumarins were used as external standards to plot the calibration curve for determination of their concentration in the *Seseli* species. The analysis was carried out on a silica column (250 × 4.6 mm ID) and detected at 320 nm using diode array detector (DAD). Elution was performed in a gradient mode using *n*-hexane:ethyl acetate, and all samples and solvents were filtrated through membrane filters (0.45 μm) prior to analysis [67].

The normal-phase system has also been employed in an LC-chemometric method for the determination of (–)-anomalin and deltoin in root and aerial parts of *Seseli resinosum* Freyn et Sint. (Umbelliferae). First, the chemometric conditions were optimized by testing different mobile phases at various solvent proportions, flow rates, and detection wavelengths to obtain the best separation results. A mobile phase consisting of *n*-hexane and ethyl acetate (75:25, v/v) in an isocratic mode at a constant flow rate of 0.8 mL/min was found to be optimal for separation and determination of these compounds. Multichromatograms (Figure 20.11) for the set of compounds in the concentration range of 50–400 ng/mL were obtained using the DAD system at a selected wavelength set, (A) 300, (B) 310, (C) 320, (D) 330, and (E) 340 nm. Three LC-chemometric approaches (LC-partial least squares [LC-PLS], LC-principle component regression [LC-PCR], and LC-artificial neural network [LC-ANN]) were applied to the multichromatographic data to construct chemometric calibrations [11].

20.4.2 Reversed-Phase HPLC in Coumarin Analyses

Reversed-phase HPLC should be the method of choice for most applications. Highly durable stationary phases with variable carbon chains and perfect reproducibility make it ideal for routine analyses as well as complicated purifications. Generally, separation of neutral coumarins is possible using a C_{18} reversed-phase column eluted with water/acetonitrile or methanol/water mobile phases, their combinations, and various additives like phosphoric acid.

Alternatively, it is possible to employ tetrahydrofuran (THF) or acetic acid as additives; these are well suited for the separation of phenolic coumarins by stepwise elution with an increasing concentration of aqueous methanol [5]. Analyses of these compounds look very promising regarding

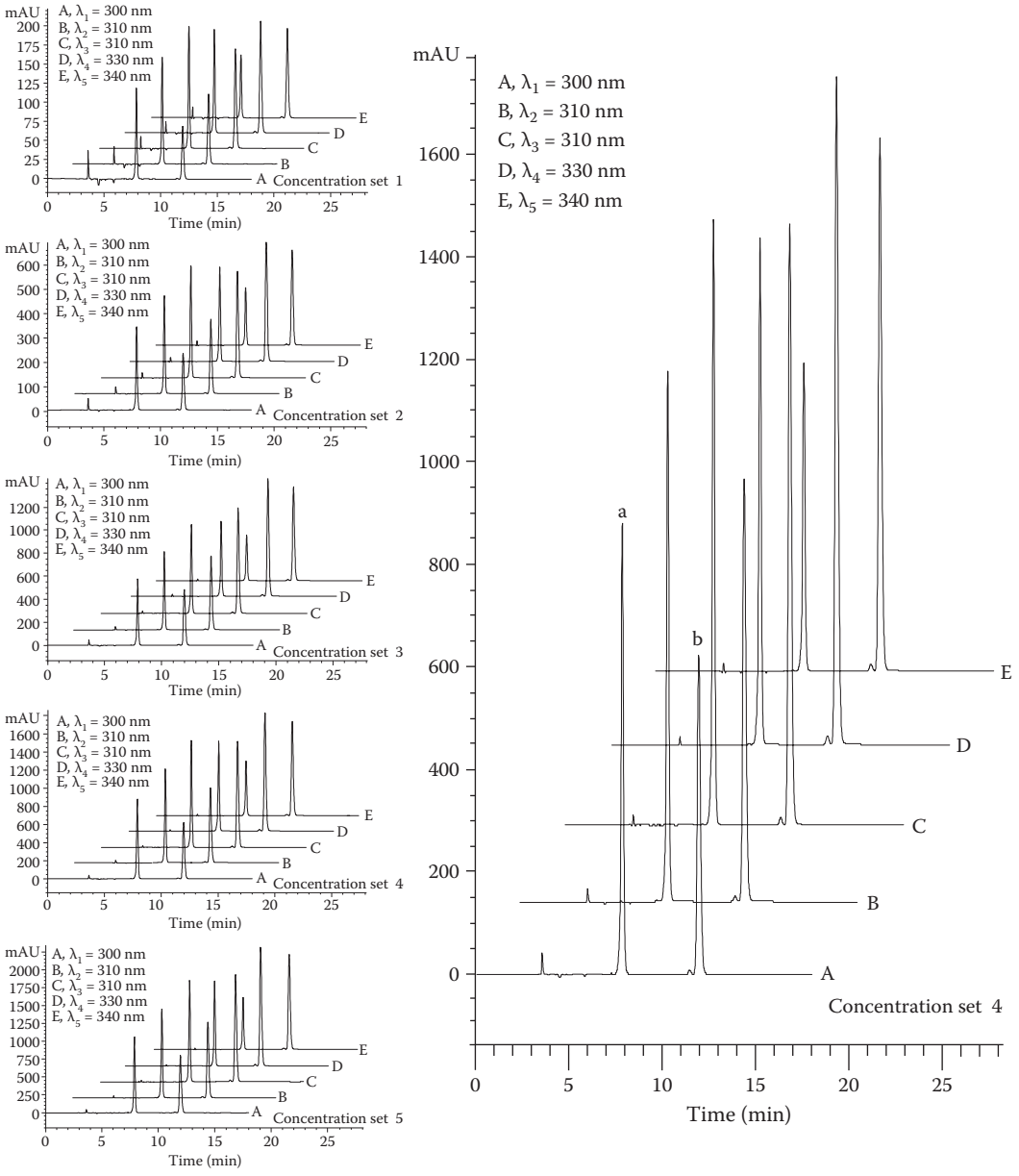

FIGURE 20.11 Multiple chromatograms of 300 ng/mL anomalin (left) and deltoin (right) in the concentration set 4. Small multiple chromatograms correspond to the concentration set 1–5. (Reprinted from Tosun, A., Bahadır, Ö., and Dinç, E., *Chromatographia*, 66, 677–683, 2007. With permission from Bontelme, Vieweg-Publishing, Germany.)

quality assurance of fruit juices [68]. Even the simple isocratic MeCN (acetonitrile): water solvent system can be of good use if employed correctly. Furocoumarins isolated from acetone extracts of dried fruits of *Tetradium daniellii* were analyzed using a LiChrospher column (250 × 4.0 mm ID, 5-μm particle size) employing this gradient at a 1 mL/min flow rate [52].

Prior to separating some simple coumarins like esculin, esculetin, fraxin, and fraxetin from *Cortex Fraxini* extracted into 50% ethanol, chloroform, ethyl acetate, or *n*-butanol, filtering is recommended. A C_{18} column (100 × 4.6 mm, 3 μm) eluted with a mixture of acetonitrile, methanol,

and 0.01% phosphoric acid (2:1:12, v/v/v), with detection at 340 nm, should then be employed for successful separation [58].

Even temperatures higher than standard can be of assistance, as in the case of the crude ethyl acetate extracts of *Notopterygium forbessi* Boiss. Those were analyzed by HPLC equipped with a UV/VIS photodiode array detector using a shim-pack CLC-ODS column (150 × 4.6 mm ID) temperate to 40°C. The mobile phase, consisting of methanol:MeCN:water (30:30:40, v/v/v), was used to elute the column in isocratic mode at a flow rate of 1.0 mL/min. Notopterol and isoimperatorin were determined to be major components in this plant besides the other unknown compounds [30].

Mobile phases containing methanol:MeCN:water in various proportions seem to be a fruitful combination of solvents, with methanol on the on hand as a perfect mildly acidic solvent and MeCN on the other, contributing to a system with better resolution and reducing backpressure at the same time. There is no wonder it was successfully used for separating coumarin constituents in the methanol extract of *Fructus Cnidii* collected from different origins in China [69]; in this specific case, the ratio of the individual solvents was 22:70.21.

In contrast, some simple furocoumarins occurring in several Umbelliferae genera such as *Heracleum*, *Angelica*, and *Peucedanum*, investigated on a reversed-phase system with DAD were found to possess similar chromatographic behavior, making them very difficult to distinguish from each other [70]. It is interesting that furocoumarins isolated from *Casimiroa edulis* Llave et Lex. did not exhibit such problems [71]. Separating compounds belonging to the same category (and thus chemically similar) can be difficult in some cases, but segregating furocoumarins from pyranocoumarins is a relatively easier task [25,72].

Efficient simultaneous chromatographic separations and quantitative analyses of 24 fragrance allergens including coumarin were achieved using conventional reversed-phase HPLC coupled with DAD. For this purpose, *p*-anisaldehyde was used as an internal standard, and a C_{18} column (250 × 4.6 mm ID) combined with a guard column was eluted isocratically with an acetonitrile:water mobile phase, with flow rates in the range 0.7–1.0 mL/min. The DAD was scanning in the wavelength range 190–500 nm. This method was used for representation of commercially available scented products. Quantitative assays were performed by means of the internal standard procedure. The calibration graphs for each standard were constructed from triplicate injections of five solutions with different concentrations and plotting the analyte against the internal standard peak areas [73].

Specific fluorescence—the unique natural trait of coumarins—can be harnessed for our cause in HPLC applications as well. Use of a fluorometric detector instead of DAD was proved to work well for the determination of 7-hydroxycoumarin (umbelliferone), which is a product formed via coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation by P450 enzymes. It was carried out using HPLC equipped with a C_{18} 5 μm analytical column (150 × 4.6 mm ID), supplemented with a C_{18} 5 μm guard column. The flow rate was 1.2 mL/min, and excitation and emission wavelengths on the detector were 338 and 458 nm, respectively [74].

There is even one peculiar application to add to the list—the use of reversed-phase HPLC for the evaluation of bioassays. The pharmacokinetics of scoparone (an anti-asthmatic coumarin derivative from *Artemisia scoparia* Waidst. et Kit), oxypeucedanin hydrate, and byak-angelicin from *Angelica dahurica* was measured using reversed-phase systems. In the case of scoparone, analytes were detected in rabbit plasma [60], and oxypeucedanin hydrate and byak-angelicin in mongrel dog plasma [75].

20.4.3 Preparative HPLC Methods in Coumarin Analyses

For the fractionation and purification of coumarins from *Citrus sinensis* extract, medium pressure liquid chromatography (MPLC) was employed using silica gel (40–63 μm) or Lichroprep 60 RP-18 (40–63 μm) columns eluted with a *n*-hexane:AcOEt (ethyl acetate) gradient with UV detection set

to 254 and 366 nm [39]. Moreover, some coumarins were purified using preparative HPLC with an inersil 10 μm ODS (250 × 22 mm) column eluted with methanol:water (85:15, v/v) at a flow rate of 12 mL/min [76]. For purification of coumarin compounds from ethyl acetate extract of *Angelica edulis*, a fraction eluted by *n*-hexane:ethyl acetate (3:1) as the mobile phase, with NP-HPLC using a silica column and detection at 320 nm, was successfully combined with recrystallization [44].

20.5 HYPHENATED HPLC TECHNIQUES IN COUMARIN ANALYSES

The LC-MS technique is becoming increasingly popular; in particular, the introduction of atmospheric pressure chemical ionization (APCI) has dramatically influenced the possibilities for analyzing poorly ionizable compounds. The use of hyphenated techniques such as LC-MS provides great information about the content and nature of constituents of complex natural matrices prior to fractioning and carrying out biological assays.

Moreover, MS presents a great advantage not only in its ability to measure accurate ion masses but also in its use in structure elucidation. This requires the structure to be fragmented either in the ion source during ionization (e.g., EI, APCI) or by capturing ions in an auxiliary device called an ion trap. In both cases, we are modulating voltage. Using EI, we could fragment only once with no control of the process, but in the case of an ion trap connected to the magnetic analyzer, in which we are able to select individual ions for fragmentation and to continue until there is enough of them, there is a theoretically limitless possibility of fragmentation. In real practice, the ion trap is limited to MS^6. This makes an ion trap a very sophisticated and powerful tool in qualitative analysis, not only for coumarins. Recently, the on-line coupling of HPLC with NMR is also being used.

The main problems of these techniques are their relative costliness and the interpretation of, in some cases, very complicated spectra, which also depends on the choice of the ionization mode. There is no single ionization mode that allows the interpretable analysis of all the constituents present in natural sources.

Particle beam (PB), thermospray (TSP), and matrix-assisted laser desorption ionization (MALDI) interfaces are the ones most commonly used for analysis of natural components, but they are suitable only for a restricted number of samples, optimization of their ionization parameters is difficult, and they can lack sensitivity in some applications.

The ESI and APCI techniques, which operate under atmospheric pressure, seem to be very promising according to some studies. Especially APCI is very useful because of its high-solvent flow rate capability, sensitivity, and response linearity. However, ESI is the technique of choice for polar and higher-molecular-weight compounds, while APCI is suitable for compounds that are less polar and lighter [77].

20.5.1 Examples of HPLC-ESI-MS

In a number of cases LC-MS provides more accurate results because of its much lower detection threshold compared to DAD. Thus, thanks to LC-MS, we are able to do both quantification and determination in a single step, as in the case of vanilla extract, which is one of the most widely used flavoring ingredients in food and beverages. Therefore, the HPLC-MS method was developed for the identification of coumarin, vanillin, and ethyl vanillin in vanilla products. Samples were analyzed using LC-ESI-MS equipped with an ODS C_{18} (250 × 2.0 mm) column eluted by acetonitrile:water (35:65, v/v) with addition of 0.1% formic acid for better ionization for 13 min at a flow rate of 0.25 mL/min, followed by a gradient of 80% acetonitrile to wash the column. UV signals were recorded at 254 nm. The MS signals were collected in both the scan and selected ion monitoring mode [78].

There is also possibility of distinguishing toxic and nontoxic chemotypes of various plants. For example, Arnoldi et al. used the different fingerprints acquired from HPLC-ESI (APCI)-DAD-MS

in both negative and positive modes to recognize toxic variants of *Ferula communis* in nature [79].

HPLC-DAD coupled with ESI-MS (HPLC-DAD-ESI-MS) was used for the simultaneous determination of coumarins present in methanolic extracts of *Angelica gigas* root. Extracts were analyzed and quantified by reversed-phase HPLC coupled with DAD, using a C_{18} column eluted by a gradient consisting of acetonitrile:water at a flow rate of 1.0 mL/min. Some of the coumarins could not be quantified by DAD because of the bad resolution of the peaks. For this reason, identification of these compounds was accomplished by ESI-MS/MS) in selected ion monitoring/selected reaction monitoring mode [47].

A plethora of studies have proved that HPLC-DAD-ESI-MS is a powerful tool for the rapid identification and determination of various components of extracts, highly specific and capable of detecting trace concentrations and providing molecular mass information. In one of the studies, *trans*- and *cis*-isomers of 2-glucosyloxycinnamic acids (with similar structure) and coumarin derivatives of their biogenic metabolites such as scopoletin, scoparone, and ayapin in *Dendrobium thyrsiflorum* were analyzed by HPLC-DAD-ESI-MS/MS. The method was carried out on a Polaris C_{18} column with a gradient solvent system (0.5% acetic acid aqueous solution in acetonitrile). The compounds were identified and quantified by comparing their retention times and their UV (342 nm) and MS spectra with authentic standards [80].

20.5.2 Examples of HPLC-APCI-MS

Due to its better results in analyses containing nonpolar or poorly ionized structures, HPLC-APCI-MS is the method of choice for the oxygen heterocyclic compounds (coumarins and polymethoxylated flavones) present in nonvolatile residues of the essential oils of mandarin, sweet orange, bitter orange, bergamot, and grapefruit. Besides the presence of clearly recognized molecular ions in the spectrum, it is possible to elucidate the structure by modulating the ionization voltage as well. One study demonstrated that in nearly all cases, the method allows precise confirmation of the predicted main components. UV and MS data were acquired and processed using software for Windows NT. MS characteristics of coumarins were determined on the basis of the response obtained with the APCI interface to characterize the citrus oil [77].

20.6 CONCLUSION

Coumarins have been isolated from many natural sources as an interesting group of compounds featuring a wide array of biological activities and structures. They can occur as simple derivatives as well as complex structures such as coumarinolignans and dimeric coumarins. Their fluorescent properties could be identification factors in many applications. Coumarins and their derivatives are able to inhibit the proliferating activity of many tumor cells by various mechanisms. These mechanisms will be important for the further development of clinical applications of these compounds. Clinically, it was proved that coumarin has a valuable action in the treatment of lymphoedemas and of burns and also, most important, anti-coagulative activity. Moreover, some products containing coumarin such as Caye Balsam, Esberiven, Flebotrat, Lodema, Venolat, Venium, Cycloarthrin, Lymphex, Micotox, and Theokal [81] are available in distribution as well as other products based on the structure of esculin.

Various analytical approaches exist for detection of coumarins. In general, the analytical technique should meet the following prerequisites: short time, relatively inexpensive, highly accurate, and precise for a variety of applications. The method of choice depends on the particular attributes and classification of the coumarins being studied.

Hopefully, this chapter can assist in the determination of coumarins of different structures using analytical HPLC techniques either for the beginning researcher or for adept researchers simply willing to extend their insight into coumarin problematics.

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21 HPLC of Flavonoids

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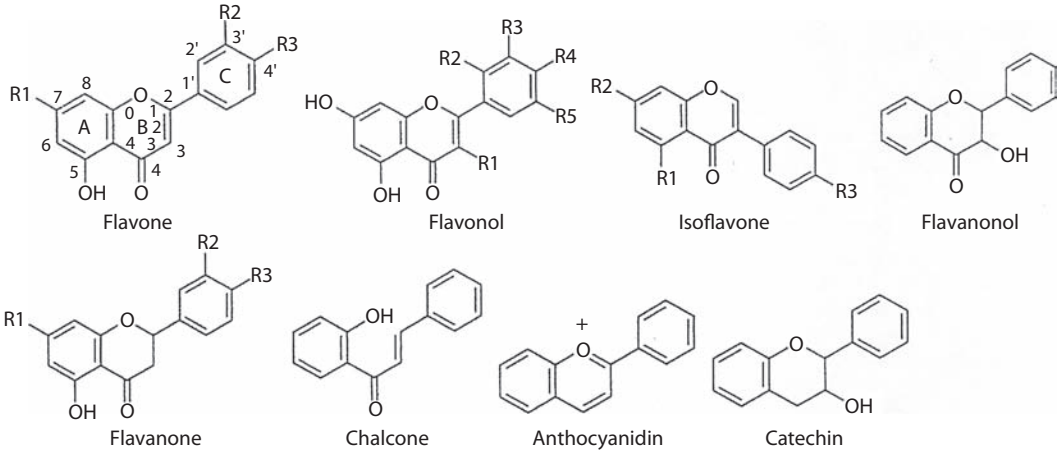
CONTENTS

21.1 DEFINITION, CHEMICAL CONSTRUCTION, PHYSICOCHEMICAL PROPERTIES, AND OCCURRENCE OF FLAVONOIDS

Flavonoids are a large group of phenolic plant constituents. To date, almost 6500 different flavonoids have been identified. These compounds consist of two benzene rings (A and C) that are connected by an oxygen-containing pyrene ring (B). Therefore, flavonoids can be regarded as chromane derivatives with a phenyl substituent in the C-2 or C-3 position. Flavonoids are often hydroxylated in positions 3, 5, 7, 3′, 4′, and/or 5′. Frequently, one or more of these hydroxyl groups is methylated, acetylated, prenylated, or sulfated. Flavonoids are mainly present in plants as *O*- or *C*-glycosides. Aglycones (the forms lacking sugar moieties) occur less frequently. At least eight different monosaccharides or combinations of these (di- or trisaccharides) can bind to the different hydroxyl groups of the flavonoid aglycone. The large number of flavonoids is a result of the many different combinations of flavonoid aglycones and these sugars. The most common sugar moieties include d-glucose, l-rhamnose, galactose, and arabinose. The glycosides are usually *O*-glycosides, with the sugar moiety bound to the hydroxyl group at the C-3 or C-7 position, whereas the *C*-glycosides have sugar groups bound to a carbon (usually



SCHEME 21.1

6-C or 8-C) of the aglycone [1,2]. Many flavonoids can be found in monomer, dimer, or polymer forms, called monoflavans, biflavans, and triflavans, respectively [3].

Flavonoids are divided into several subgroups, and it is important to keep in mind that the biological and chemical properties of flavonoids belonging to different subgroups can be quite different. The most frequently encountered classification of flavonoid aglycones includes flavones, flavonols, flavanones, flavanonols, isoflavones, anthocyanidins, chalcones, catechins, and biflavonoids. The structures and main subclasses of flavonoids are depicted in Scheme 21.1 and Table 21.1 [1]. Isoflavonoids differ from the other groups; the B ring is bound to C-3 of ring C instead of C-2. Anthocyanidins and catechins, in contrast, lack the carbonyl group on C-4, while the basic structure of chalcones has no middle heterocyclic ring.

Flavonoid aglycones possess the chemical properties of phenolics, and thus they are slighty acidic. Those possessing a number of unsubstituted hydroxyl groups or sugar moieties are polar substances and soluble in polar organic solvents (e.g., water, ethanol). The presence of sugar makes flavonoids more water soluble, while less polar aglycones like isoflavones, flavanones, and the highly methoxylated flavones and flavonols tend to be more soluble in ether or chloroform [4,5].

The main flavonoids and their sources are listed in Table 21.2. Flavones are not frequently found in fruits but are found in grains, some herbs, and some vegetables. Common flavones are luteolin and apigenin. The best-known flavonols are quercetin and kaempferol, which are most common among fruits and vegetables (some berries, herbs, legumes, and root and leafy vegetables). Chickpeas, cumin, hawthorn berry, licorice, peppermint, rowanberry, and citrus fruits are among the few plants that contain flavanones (e.g., hesperidin, narirutin, and their glycosides). Isoflavonoids are found predominantly in legumes. Soybeans are the major source of daidzein and genistein, which are also found in black beans, green split peas, and clover sprouts. Anthocyanins produce the blue and red coloration of berries, cherries, plums, eggplant, red cabbage, and radishes. The anthocyanin color is pH dependent. These compounds often occur in a complex mixture and may be complexed with flavones or metal ions such as iron and magnesium in flowers [2,6].

21.2 BIOLOGICAL MEANING AND PHARMACOLOGICAL ACTIVITIES OF FLAVONOIDS

Flavonoids have received considerable attention in the literature, specifically because of their biological and physiological importance. They play an important role in plants as defense and signaling

TABLE 21.1
Main Flavonoid Subclasses

Flavonoid Subclasses	R_1	R_2	R_3	R_4	R_5
		Flavonol			
Rutin	3-*O*-Rha-glu	H	OH	OH	H
Kaempferol	OH	H	H	OH	H
Quercetin	OH	H	OH	OH	H
Morin	OH	OH	H	OH	H
Isorhamnetin	OH	H	OCH_3	OH	H
Myricetin	OH	H	OH	OH	OH
Fisetin	OH	H	H	OH	OH
		Flavanone			
Hesperetin	OH	OH	OCH_3		
Hesperidin	7-*O*-Rha-glu	OH	OCH_3		
Naringin	7-*O*-Rha-glu	H	OH		
Naringenin	OH	H	OH		
Eriocitrin	7-*O*-Rha-glu	OH	OH		
Eriodyctiol	OH	OH	OH		
Isosakumatin	OH	H	OCH_3		
		Flavone			
Chryseoriol	OH	OCH_3	OH		
Chrysin	OH	H	H		
Apigenin	OH	H	OH		
Luteolin	OH	OH	OH		
Acacetin	OH	H	OCH_3		
Genkwanin	OCH_3	H	OH		
		Isoflavone			
Biochanin A	OH	OH	OCH_3		
Sissotrin	OH	7-*O*-Glu	OCH_3		
Genistein	OH	OH	OH		
Genistin	OH	7-*O*-Glu	OH		
Formononetin	H	OH	OCH_3		
Ononin	H	7-*O*-Glu	OCH_3		
Daidzein	H	OH	OH		
Daidzin	H	7-*O*-Glu	OH		

Source: Adapted from de Rijke, E., Out, P., Niessen, W.M.A., Ariese, F., Gooijer, C., and Brinkman, U.A.T., *J. Chromatogr. A*, 1112, 31–63, 2006. With permission.

compounds in reproduction, pathogenesis, and symbiosis. Plant flavonoids are involved in response mechanisms to stress, as caused by elevated UVB radiation, infection by microorganisms, or herbivore attack. Flavonoids are also involved in the production of root nodules as a nitrogen fixation system after infection by *Rhizobium* bacteria in leguminous plants and are pigment sources for flower coloring [1].

Flavonoids also affect human and animal health because of their biological and pharmacological activities. They are common components of remedies. Rutin and diosmin preparations are commonly used to increase vascular tone. Anthocyanins from *Vaccinum* and *Ribes* (such as bilberry or black currants) are used to enhance vision and increase capillary resistance [6].

TABLE 21.2
Main Flavonoids and their Source in Foods

Flavonoid	Source
Flavonol	
Quercetin-3,4′-glucoside	Onion
Quercetin-3-glucoside	Onion, apple
Quercetin-3-rhamnoglucoside (rutin)	Onion, apple
Quercetin-3-galactoside	Black tea, black currant
Quercetin-3-rhamnoside	Apple, black currant
Quercetin-3-arabinoside	Apple
Myrisetin-3-glucoside	Black currant
Flavonols	
(+)-Catechin	Apple, red wine
(−)-Epicatechin	Apple, red wine
(Epi)catechin and their gallates	Black tea
Flavanone	
Hesperetin-7-rhamnoglucoside (hesperidin)	Orange juice
Naringenin-7-rhamnoglucoside (narirutin)	Orange juice, grapefruit juice
Naringenin-7-rhamnoglucoside (naringin)	Grapefruit juice
Flavone	
Luteolin-7-apiosylglucoside	Red pepper
Isoflavones	
Genistein-7-glycoside	Soybeans
Daidzein-7-glycoside	Soybeans
Anthocyanins	
Cyanidin-3-rutinoside	Black currant
Cyanidin-3-glucoside	Black currant
Delphinidin-3-rutinoside	Black currant
Delphinidin-3-glucoside	Black currant

Source: Adapted from Erlund, I., *Nutr. Res.*, 24, 851–874, 2004. With permission.

Several classes of flavonoids were investigated for their antiproliferative activity against human cancer cells [7–10]. The flavonoid-rich fresh fruit juices extracted from several *Citrus* species showed antiproliferative activity (against human chronic myelogenous leukemia and human breast adenocarcinoma) [11]. Flavonoids, the chief ingredients of radix *Scutellariae baicalensis*, are well known for their anti-inflammatory and antiallergenic activities. Furthermore, *Scutellaria baicalensis* has antitumor activity. Some flavonoids present in this plant inhibit the development of skin tumors and prostate cancer cells. Herba *Scutellaria rivularis* has been used for the treatment of hepatitis, liver cirrhosis, cancers of the digestive system, hepatoma, lung cancer, breast cancer, and chorioepithelioma. For that reason *Scutellaria* species have been important medicinal plants in traditional medicine in China and Japan [12]. Also, *Cudrania tricuspidata* (Carr.) Bureau is an important traditional herbal remedy in East Asian countries such as Korea, China, and Japan. Root bark of this species contains biologically active flavonoids. Extracts of root bark exhibit antibacterial, antioxidant, cytotoxic, hypotensive, and anti-inflammatory activities [13]. Flavonoids isolated from *Caesalpinia pulcherrima* L. also have anti-inflammatory properties. These compounds significantly and dose-dependently inhibited the inflammatory mediators

[14]. Two main flavonoid glycosides, kaempferol-3,7-*O*-α-dirhamnoside and quercetin-3,7-*O*-α-dirhamnoside, isolated from *Tilia argentea* Desf. ex DC leaves [15] as well as flavonoid fractions extracted from *Zizyphus lotus* [16] and from *Caralluma attenuata* [17] possess antinociceptive and anti-inflammatory activities. The flavonoid-rich extract from the capitula and the leafy stems of *Helichrysum compactum*, containing apigenin, kaempferol, luteolin, naringenin, 3,5-dihydroxy-6,7,8-trimethoxyflavone, kaempferol-3-*O*-glucoside, luteolin-7-*O*–glucoside and luteolin-4′,7-di-*O*-glucoside, quercetin, apigenin-7-*O*-glucoside, and quercetin-3-*O*–glucoside, shows antioxidant activity by inhibition of lipid peroxidation and also demonstrates antibacterial activity [18]. Flavonoids isolated from *Teucrium polium* L. (mainly rutin and apigenin) also have antioxidant and free radical–scavenging properties [19]. This group of compounds is helpful in the fight against viral [20,21], bacterial [18,22], and parasitic diseases [23]. Other important activities of flavonoids are sealing and augmentation of capillary blood vessels and antithrombotic [3,24,25], cardioprotective [26], hepatoprotective [27], and gastrointestinal protective properties [28]. For these reasons flavonoids are widely used in therapy.

21.3 ANALYTICAL METHODS OF FLAVONOID ISOLATION

21.3.1 Extraction from Plant Material

The extraction yield of flavonoids from plant materials is influenced by their chemical nature (e.g., polarity, acidity). The extraction method employed depends on sample particle size, storage time and conditions, and the presence of interfering substances. Common solvents for extractions of these compounds are hot water, acetonitrile, ethyl acetate, ethanol, methanol, and aqueous methanol, the latter being the most common. The most popular extraction methods used for isolation of flavonoids from plants are Soxhlet extraction, ultrasonification (also known as ultrasound-assisted extraction [USAE]), microwave-assisted solvent extraction (MASE), accelerated solvent extraction (ASE), which is also called pressurized liquid extraction (PLE), and supercritical fluid extraction (SFE) [1,29].

Soxhlet extraction using methanol (12 h) gives the best extraction results for various flavonoids isolated from *Hypericum perforatum*, *Urtica dioica*, *Tilia europea*, and *Mentha spicata* [30]. In contrast, aqueous ethanol gives the best yields of flavonoid glycosides extracted from *Ginkgo biloba* leaves [1].

Exhaustive extraction in Soxhlet apparatus was the most efficient method of isolation for rutin and isoquercitrin from *Sambucus nigra* L. inflorescence compared to other methods used (USAE, MASE, ASE). Two grams of each plant material was placed in filter paper and then in the thimble holder of the apparatus and extracted for 15 h using methanol [29]. In addition, USAE has been used to extract bioactive compounds from *Sambucus nigra* L. inflorescence and from *Polygonum aviculare* herb. Isoquercitrin from *Sambucus nigra* L. is well extracted by USAE at 60°C. For rutin extracted from *Polygonum aviculare* herb, ultrasound- as well as microwave-assisted methods give a significantly higher yield than long-lasting exhaustive Soxhlet extraction [29]. USAE was found to be fast and reliable and gave better results than the mix-stirring technique for the extraction of isoflavone glucosides from soybeans. Large amounts of isoflavones are extracted with ethanol (50%, 10 and 20 min, 60°C) [31] and acetonitrile in water (1:1, v/v) [32].

Extraction of the flavonoids from red grape skins was performed by ultrasonication, using hydrochloric acid in methanol as the extraction solvent. The samples were immersed in an ultrasound bath and subjected to ultrasound treatment at 25°C and a constant frequency of 35 kHz during 15 min. After that the samples were taken out of the ultrasound bath and left at room temperature for 1 h. The extract was centrifuged at 3500 rpm for 20 min, collected in a the laboratory flask, and stored in the refrigerator. The remaining solid skins were extracted two more times. Finally, the three portions of grape skin extracts were mixed, and the total volume of the extract was measured. The total ultrasound exposure time of the grape skins, after the three consecutive

extractions, was 60 min at a frequency of 35 kHz, a frequency commonly used in extraction. In this way the extraction time was significantly reduced compared with other traditionally used extraction techniques [33].

USAE with methanol or aqueous methanol was an effective method of extraction of bioactive flavonoids from the rhizome of *Alpinia officinarum* (15–20°C, 60 min) [34]; of epicatechin, catechin, rutin, apigenin, luteolin, and quercetin from *Ginkgo biloba* leaves (30 min) [35]; and of isoflavone and flavonol-glucoside-(di)malonates from *Trifolium pratense*, *T. dubium*, and *T. repens* leaves (20°C) [36]. USAE using acetone with water (7:3, w/w) gave a good yield of epicatechin, catechin, procyanidin, flavonols, anthocyanins, and dihydrochalcones. Wen Huang et al. optimized USAE of flavonoids from *Folium eucommiae*. The results indicate that the highest extraction percentage of flavonoids by USAE could reach 17.2% using 40% ethanol as the solvent, which was more efficient than that achieved by heating, microwave-assisted, and enzyme-assisted extraction methods [37].

Another study proved that using the ultrasonic method should be the most economic way to enhance the extraction yield of an isoflavone-containing herbal extract in a short time with a reduced amount of solvent at a lower temperature. In this study the application of three extraction methods (traditional, pressurized solvent extraction, and ultrasonic techniques) for preparing ethanolic *Radix Puerariae* extract was demonstrated. A comparison of the three extraction methods showed preferential higher yields of the three major isoflavones (puerarin, daidzin, and daidzein) when the ultrasonic technique was applied [38].

A microwave-assisted extraction technique was developed for the fast extraction of flavonoids from *Radix astragali*. Several influential parameters of the MASE procedure (microwave power, extraction cycles, ethanol concentration, extraction temperature, irradiation time, and solvent-to-material ratio) were studied for the optimization of the extraction protocol. The maximum yield of flavonoids with MASE was obtained by dual extraction with 90% ethanol and 25 mL/g material at 110°C for 25 min. No degradation of the flavonoids was observed using the developed extraction protocol. The optimal yield with MASE was close to that of Soxhlet extraction with methanol for 4 h and higher than that of USAE with methanol for 2×30 min and heat reflux extraction with 90% ethanol for 2×2 h [39]. High yields of rutin and isoquercitrin were obtained in microwave-assisted extraction using an open system (80% methanol in water) in the case of *Sambucus nigra* L. inflorescence and *Polygonum aviculare* herb [29]. The dynamic microwave-assisted extraction (1200 W of radiation power, 80% ethanol, flow rate of 50 ml s^{-1}) showed obvious advantages in its short duration and high efficiency for extracting flavonoids without causing degradation of target components from *Saussurea medusa* Maxim, in comparison with dynamic solvent extraction without microwave assistance [40].

ASE (also known as PLE) was an effective method for extraction of rutin and isoquercitrin from *Sambucus nigra* L. [41] and *Polygonum aviculare* herb. In the second case this method (methanol, 100°C, 60 bar) gives the best results in comparison to other methods used [29]. In another investigation, the extraction of flavonoids from *Houttuynia cordata* Thunb by PLE was studied. The effects of several important factors such as the concentration of ethanol in the solvent, flow rate, temperature, and pressure were investigated. A high flavonoid yield was obtained with a solvent ethanol concentration of 50%, flow rate of 1.8 mL/min, temperature of 70°C, and pressure of 8 MPa. The results are favorable to that of traditional methods of hot soaking and USAE [42].

Papagiannopoulos et al. applied on-line coupling of ASE, solid-phase extraction (SPE), and high performance liquid chromatography (HPLC) for automated analysis of proanthocyanidins in malt. ASE is suitable to extract 99% of the proanthocyanidins. Acetone–water (80:20, v/v) was found to be the optimal extraction solvent. A temperature lower than 60°C results in inefficient extraction, and a higher temperature shows analyte loss due to thermal degradation. However, using 60°C, no thermal degradation during the extraction was observed. A pressure setting of 100 or 200 MPa showed no difference. The total time for a single sample extraction is approximately 25 min compared to 2 h with the manual method [43].

Another useful technique for the isolation of flavonoids from plant material is SFE. Three flavonoids including orotinin, orotinin-5-methyl ether, and licoagrochalcone B from the traditional Chinese medicine *Patrinia villosa* Juss were extracted by SFE. Under optimal conditions—that is, a pressure of 25 MPa, a temperature of 45°C, a sample particle size of 40–60 mesh, and a modifier of 20% methanol —high extraction yields of flavonoids were achieved [44].

Another recent application of SFE is extraction of flavonoids from *Ginkgo* leaves. Analytical results demonstrated that three dry supercritical CO_2, N_2O, and could not extract *Ginkgo* flavonoids. Because of their medium to high polarity, flavonoids require a long extraction time as well as cosolvent addition. When two ratios of cosolvents (ethanol) were individually preloaded with *Ginkgo* leaves in the extractor, the amount of flavonoids was significantly increased [45].

Supercritical carbon dioxide extraction was employed to extract flavonoids from *Pueraria lobata*. The optimal conditions to obtain the highest flavonoid yield of *P. lobata* were a pressure of 20.04 MPa, a temperature of 50.24°C, and a cosolvent (ethanol) amount of 181.24 mL [46]. SFE gives good results in extraction of naringin from *Citrus paradise* (9.5 MPa, 58.6°C) and epicatechin from sweet Thai tamarind seed coat [47] as well as baicalin, baicalein, and wagonin from *Scutellaria baicalensis* radix. In the latter case, optimal extraction conditions were as follows: supercritical carbon dioxide–methanol–water (20:2.1:0.9), 50°C, 200 bar. The extraction efficiency was better than in the case of USAE [48].

Flavonoids may exist in the plant as free aglycones or glycosides. If aglycones are the target analytes, chemical hydrolysis is usually performed. Two main procedures to cleave the glycoside bond are reported in the literature: acid hydrolysis and enzymatic hydrolysis. More often, the acid hydrolysis of flavonoid glycosides is performed—with hydrochloric acid or formic acid at elevated temperatures (80–100°C) or by refluxing with acid in the presence of ethanol. Complete acid hydrolysis of phenolic glycosides gave 6,8-di-C-glucosylapigenin or vicenin and glucose. These compounds were hydrolyzed with aqueous methanolic hydrochloric acid (1:1) at 100°C for 7 h. Complete acid hydrolysis of flavonol glycosides afforded kaempferol or quercetin, glucose, and galactose. Each compound was treated with hydrochloric acid (1 h reaction time, in a sealed vial, at 100°C), and, after cooling, the reaction mixture was extracted with diethyl ether [49–51]. If the interest is in the intact flavonoid glycosides, hydrolysis should of course be prevented. This means that harsh extraction conditions and heating should be avoided. Furthermore, the activity of hydrolyzing enzymes that may be released during milling of plant material can be inhibited by addition of, for example, tris(hydroxymethyl)aminomethane [1].

21.3.2 Sample Purification and Concentration

Liquid–liquid extraction (LLE) has been developed for purification of flavonoids from *Sambucus nigra* L. inflorescence and *Polygonum aviculare* herb. All methanolic extracts (obtained by Soxhlet, USAE, MASE, and ASE methods) were evaporated to dryness. Dry residues soaked with boiling water were cooled in the refrigerator, filtered, and extracted with diethyl ether (for the isolation of phenolic acids). Then, the water solution was extracted by ethyl acetate. Ethyl acetate extracts were collected and evaporated to dryness, and the dry residues were then dissolved in methanol and flavonoids were analyzed quantitatively by reversed-phase HPLC (RP-HPLC) [29].

The SPE method is very often used in sample pretreatment for HPLC. In most cases the sorbent is C_{18}-bonded silica, and the sample solution and solvents are usually slightly acidified to prevent ionization of the flavonoids, which would reduce their retention [1]. SPE has been developed for the purification of flavonoids, such as rutin and isoquercitrin from *Sambucus nigra* L. inflorescence and *Polygonum aviculare* herb (using C_{18} sorbent and polyamide and methanol or aqueous methanol as solvent) [29]. The most efficient purification method was SPE using octadecyl sorbent. The method is characterized by high repeatability (RSD ≤ 1.12%). SPE C_{18} also gives high recoveries of analyzed flavonoids (up to 93.4% for rutin and up to 90.1% for isoquercitrin). Repeatability and

recoveries for investigated flavonoids were distinctly lower with the LLE technique. Moreover, LLE changes the proportions of rutin and isoquercitrin. In the case of SPE on polyamide, the repeatability is also relatively high (RSD ≤ 2.14%). Recoveries for rutin are also high, up to 93.27%. However, recoveries for isoquercitrin are very low—only about 40%—because polyamide strongly retained more hydrophobic compounds [29].

For the analysis of a red clover extract, a dual-SPE method was used [52]. The methanolic extract was subjected to SPE, and fractions were collected and transferred to a second SPE sorbent. Three sorbents were tested, and the pH and concentration of the aqueous organic solvent were varied. Optimum conditions were created by applying the extract to a C_{18} sorbent, washing with methanol–water (35:65, v/v) containing 2% acetic acid, and eluting with a methanol–water mixture with an organic-solvent proportion increasing from 0 to 90% and containing 2% aqueous ammonia. With the same sorbent as in the first step, in the second step a mixture of 80% methanol containing 2% aqueous ammonia was used to completely elute all analytes from the sorbent. In the extract 49 flavonoids, including several acylated flavonoid glucosides, were identified.

Purification of the proanthocyanidins extracted from malt was performed using an ASPEC (automated sample preparation with extraction cartridges) system directly coupled to the ASE for automated transfer of the extracts to the SPE cartridges [43]. ASPEC is a sampler for automated SPE and serves as an autosampler for injection in liquid chromatography (LC) systems. The ASPEC system performed the following tasks after completion of the extraction by ASE: The SPE polyamide cartridges were conditioned with water. In the ASE collection vial, the crude extracts were diluted with water and mixed in liquid mode by aspirating and dispensing of the diluted extract. The extracts were loaded onto the SPE cartridge quantitatively, the liquid pushed through the cartridge with air, and the cartridge washed with water and dimethylformamide (DMF)–water (85:15, v/v). Finally, the adsorbed analytes were eluted with 2.5 mL of the latter solvent.

The use of matrix solid-phase dispersion (MSPD) was tested, separately, for the extraction of phenolic compounds and organic acids from white grapes. This method was compared with a more conventional analytical method that was previously developed. The sample was weighed, mixed, and blended carefully with C_{18} sorbent in a mortar to obtain a mixed stationary phase. Then, the mixture was introduced into a 15-mL syringe body and packed using a syringe plunger. After the elution of organic acids and other polar compounds with aqueous solvent, the retained phenolic fraction was eluted with ethanol. This extract was taken to dryness under reduced pressure (30°C) and redissolved in methanol (1 mL), and then 20-µL portions were analyzed by HPLC–diode array detection (DAD). Recoveries obtained for phenolic compounds were, in general, lower for MSPD than for solid–liquid extraction (SLE)–SPE. Only the recoveries obtained for the pair quercetin-3-*O*-glucoside/quercetin-3-*O*-rutinoside and for epicatechin were similar, in some assays, to those obtained using SLE-SPE. SLE-SPE is a conventional analytical method in which SLE is followed by SPE. However, a very high volume of solvent, not usual in MSPD, was necessary. Recoveries of quercetin and kaempferol, in all the assays carried out by MSPD, were always much lower than by SLE-SPE, with the best recoveries being only around 30% [53].

Isoflavonoids from *Radix astragali* were extracted effectively by MSPD-C_{18} and then determined and identified by HPLC-DAD-MS (mass spectrometry). MSPD was compared to Soxhlet and ultrasonic extraction. In this method, the column was packed in an extraction layer and a separation layer to increase the separation efficiency. The results for the fraction collected from two-layer columns indicated that the two-layer method was better than the homogeneous packing method. Several different solvents with good solubility for isoflavonoids, including acetone, ethyl acetate, methanol–water, and acetronitrile–water, were investigated. The best result was obtained with 90% aqueous methanol elution. The MSPD method exhibited acceptable reproducibility, recovery, extraction efficiency, and consumption (of sample, solvent, and time) relative to conventional extraction techniques such as ultrasonic and Soxhlet methods [54]. A similar MSPD procedure was used to obtain analyte enrichment and sample cleanup for LC–nuclear magnetic

resonance (NMR) analysis of red clover leaves. This approach provided sufficiently high concentrations of the seven main isoflavones in these leaves to permit their identification while using a mere 500 mg of sample. In this case, MSPD-based sample preparation has the disadvantage of being somewhat more time-consuming than SLE and therefore more prone to (partial) hydrolysis in the case of flavonoid conjugates. Furthermore, compared with SPE, the extraction efficiency for the glucosides was found to be lower. Obviously, a systematic study of sorbent materials is urgently needed [1].

In the extraction of flavanones and xanthones from root bark samples of *Maclura pomifera*, SLE, MSPD with C_{18}-bonded silica, and, as a novel approach, MSPD with sea sand were used. For SLE, samples were soaked in dichloromethane or methanol–water (9:1, v/v). For the MSPD procedures, samples were mixed with the C_{18} sorbent or sea sand and hexane, packed into a column, and eluted with dichloromethane or methanol–water. The best results were obtained with the sea sand procedure, with C_{18}-MSPD in second place, and SLE last: When using sea sand, the analyte responses in LC-UV were about 25% higher than with SLE. This seems to suggest that, for this application, analyte losses due to incomplete extraction were more important than sample cleanup. In the root bark extracts, five prenylated xanthones and two prenylated flavones were found [55]. MSPD has been demonstrated to be a suitable technique for the extraction of naturally occurring compounds, for example, flavonoids, from plant material, a simple alternative to LLE and SPE.

Solid-phase microextraction (SPME) is generally combined with gas chromatography (GC) analysis for the extraction of (semi)volatile organic compound samples but has also been coupled with LC to analyze nonvolatile and/or polar compounds. SPME is a straightforward technique, and organic solvent consumption is less than in SPE. However, because it is an equilibrium method, analyte recoveries can be quite low while extraction times are frequently as long as 60 min. Mitani et al. used an open-tubular fused-silica capillary column for SPME instead of a fiber [56]. The authors determined genistein and daidzein in soybean foods using on-line in-tube SPME-LC-DAD UV. Optimum extraction conditions for standard solutions were obtained with 20 draw/eject cycles of 40 μL of sample using a porous-layer open-tubular capillary column; the total extraction-plus-desorption time was 30 min. Analyte recoveries from spiked food were above 97% in all cases. Unfortunately, compared with an earlier study [57], the limits of detection were about 50-fold higher, that is, about 0.5 ng/mL.

21.4 COLUMN LC

HPLC has been used for separation and determination of flavonoids since the 1970s [58]. Up to now many papers have been published on this subject, and HPLC of flavonoids is periodically reviewed in numerous journals connected with plant chemistry and food analysis [1,59–62]. Although recently other modern separation systems have been used for the determination of flavonoids in foods and plant material, that is, capillary zone electrophoresis [63–67] and micellar electrokinetic capillary chromatography [68], by far the most widely employed technique has been HPLC.

21.4.1 Optimization

Thin-layer chromatography (TLC), which gives the possibility of obtaining a lot of data in one run, was used to optimize the separation of flavonoids in reversed-phase systems [69]. Retention coefficients were determined for flavonoid standards on C_{18} precoated plates using aqueous mobile phases containing organic modifiers (methanol, acetonitrile, tetrahydrofuran) at different concentrations. The same systems were applied for determination of retention parameters by HPLC. Retention

parameters obtained by two techniques were correlated with each other as log k vs. R_M dependencies for every chromatographic system separately. Obtained high regression coefficients $R^2 > 0.96$ verify the possibility of using TLC experiments for optimization of separation of flavonoids prior to HPLC analysis.

21.4.2 Stationary Phases

Although flavonoids are compounds of wide structural diversity and polarity, the systems used for their separation are usually similar. The most often used columns are filled with reversed-phase C_{18} sorbent. Packings of the C_8 type have been employed for separation of more polar flavonoids, for example, aglycones and glycosides of isoflavones [70–72].

A paper by Crozier et al. [73] reports on the evaluation of the performance of five reversed-phase supports for HPLC of flavonoids. Stainless steel columns of 150–250 mm length and 3.9–4.6 mm diameter, filled with sorbents of 4 or 5 μm particles and manufactured by various producers (ODS-Hypersil [Shandon, UK], RP-18 LiChrospher [Merck, Germany], C_{18} Nova-Pak [Waters], C_{18} Symmetry [Waters], and C_{18} Genesis [Jones Chromatography, UK]), were used in experiments. The columns were eluted at flow rates ranging from 0.6 to 1 mL min^{-1} in isocratic and gradient modes with mixtures in water or methanol in water adjusted to pH 2.5 with trifluoroacetic acid. In order to assess and optimize HPLC conditions, the performance of the applied columns was investigated using benzophenone and flavonoids: rutin and its aglycone quercetin. The column performance parameters were theoretical plates (1/H) and peak tailing (T). When benzophenone was used (asolute used by manufacturers to test column efficiency), all five columns exhibited high efficiency and correct tailing factor. With rutin, both ODS-Hypersil and LiChrospher RP-18 columns exhibited a decline in efficiency and an increase in T. Analysis of quercetin revealed marked differences in the performance of the five columns. Broad, low-efficiency peaks with excessive tailing were obtained with ODS-Hypersil and LiChrospher RP-18 supports. In contrast, no deterioration of 1/H values was observed, together with minimal effect on the tailing factor, when quercetin was chromatographed on the Nova-Pak, Symmetry, and Genesis columns. The data reported in this paper demonstrate that the ODS-Hypersil and LiChrospher RP-18 columns can provide acceptable reversed-phase analyses of flavonoid glycosides. However, they are not suitable for use with free flavonoid aglycones because of high band broadening and excessive peak tailing. In contrast, high-efficiency analyses of both glycosides and aglycones were obtained with Nova-Pak, Symmetry, and Genesis columns. These Symmetry and Genesis columns were used for gradient elution analysis of a range of free and conjugated flavonoid standards. Figure 21.1 presents similar separation profiles for standards chromatographed with both columns using gradient elution in an acetonitrile-based solvent [73].

Similar investigations were performed for catechins [74]. Comparison of six reversed-phase C_{18} columns indicates that deactivated stationary phases, which utilize ultrapure silica and maximize coverage of the silica support, provide significantly improved separation and chromatographic efficiencies for catechin analyses using LC, compared to conventional monomeric or polymeric C_{18} columns [74]. Figure 21.2 shows peak profiles obtained on six different C_{18} columns, indicating that monomeric C_{18} columns based on a deactivated silica matrix are equivalent in the separation of catechins.

With the advent of the ultra performance LC (UPLC) system, the new technology is able to operate at high pressure (1000 bar, compared with 400 bar for HPLC), using 1–5 mm ID columns packed with 1–2 μm particles at high flow rates [60]. In this way, the performance of the LC technique is improved: The times for flavonoid analysis are shortened, and the resolution and the sensitivity are higher [75–77]. For example, Chen et al. developed a UPLC method for simultaneous determination of 15 flavonoids in *Epimedium* [77]. The analysis was performed on a UPLC BEH C_{18} column (50 × 2.1 mm ID, 1.7 μm) with gradient elution of 50 mM acetic acid aqueous solution

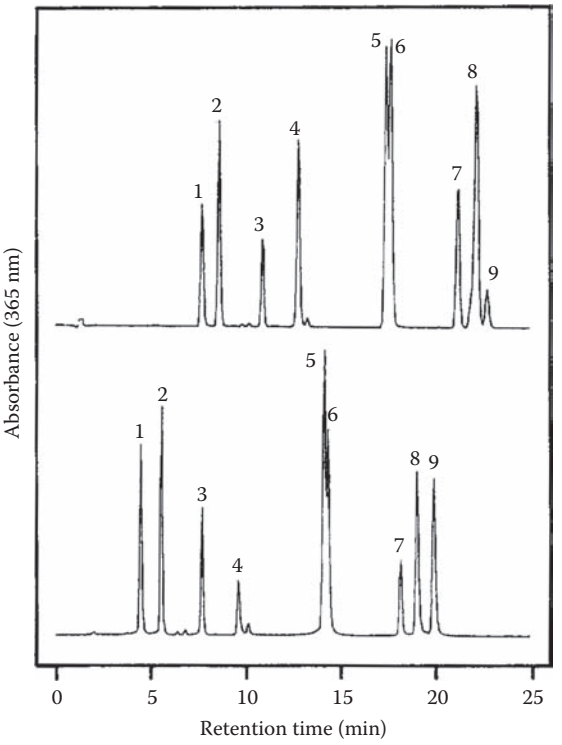


FIGURE 21.1 Gradient reversed-phase HPLC analysis of free and conjugated flavonoids. Upper trace: C_{18} Symmetry column (150 × 3.9 mm ID, 5 μm) eluted with 20-min gradient of 15–35% acetonitrile in water adjusted to pH 2.5 with trifluoroacetic acid. Lower trace: C_{18} Genesis column (150 × 4.6 mm ID, 4 μm) eluted with with 20-min gradient of 20–40% acetonitrile in water adjusted to pH 2.5 with trifluoroacetic acid. Flow rate; 1 mL min^{-1}. UV detection at 365 nm. Standards: 1 – rutin, 2 – quercetin-3-glucoside, 3 – quercitrin, 4 – myricetin, 5 – luteolin, 6 – quercetin, 7 – apigenin, 8 – kaempferol, 9 – isorhamnetin. (From Crozier, A., Jensen, E., Lean, M.E.J., and McDonald, M.S., *J. Chromatogr. A*, 761, 315–321, 1997. With permission.)

and acetonitrile within 12 min. All calibration curves showed good linearity ($R^2 > 0.9997$) within test ranges. The limit of detection (LOD) and limit of quantitation (LOQ) were lower than 0.13 and 0.52 ng on the column, respectively.

21.4.3 Mobile Phases

Generally, the mobile phases applied for reversed-phase systems are aqueous acetonitrile or methanol in combination with an acid as an ion suppressant. Sometimes the use of tetrahydrofuran and 2-propanol as the nonpolar solvent has been reported [1,73]. The last additives increase the separation selectivity of flavonoids. The greatest differences in eluent composition are observed in the type of acid used as the modifier minimizing peak tailing. Most often, addition of acetic acid, formic acid, citric acid, or trifluoroacetic acid was applied; however, acetate buffer or formate buffer at a low pH are also used.

A paper by Dalluge and coworkers shows the effect of acid in the mobile phase on the chromatography of catechins [74]. Figure 21.3 shows a chromatogram of catechin standards eluted from the reversed-phase column (deactivated, monomeric C_{18}, ultrapure silica) by the mobile-phase gradient 1 in the presence (A) and absence (B) of 0.05% trifluoroacetic acid and in the mobile phase 2 in the presence (C) and absence (D) of 0.05% trifluoroacetic acid (for gradient details, see reference 74).

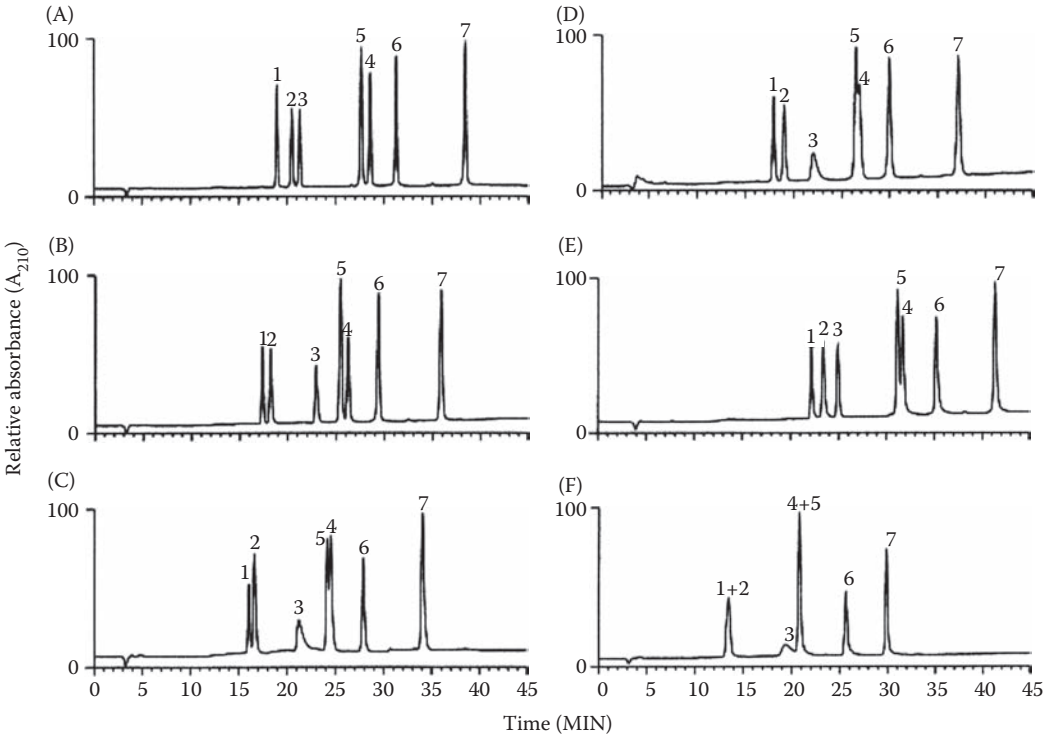


FIGURE 21.2 Comparison of six reversed-phase HPLC columns (A–F) for the chromatographic separation of six catechins and caffeine in a standard mixture using gradient elution: (A) water + 0.05% trifluoroacetic acid; (B) 60:40 methanol–acetonitrile + 0.05% trifluoroacetic acid; for gradient details, see (From Dalluge, J.J., Nelson, B.C., Brown Thomas, J., and Sander, L.C., *J. Chromatogr. A*, 793, 265–274, 1998. With permission.) The concentration of each catechin in the standard mixture was 0.05 µg/µL. Detection was carried out with UV at A_{210}. Peak identification: 1, epigallocatechin; 2, (+)-catechin; 3, caffeine; 4, epicatechin; 5, epigallocatechin gallate; 6, gallocatechin gallate; 7, epicatechin gallate. A description of each column is given below:

Column	Type	Description
A	Zorbax Eclipse XDB-C_{18} Rockland Technologies Inc./Dupont	Deactivated, endcapped monomeric C_{18}, ultrapure silica
B	Zorbax Rx-C_{18} Rockland Technologies Inc./Dupont	Deactivated, monomeric C_{18}, ultrapure silica
C	PAH Hypersil Keystone scientific Inc.	Polymeric C_{18}
D	SMT OD-5-100 Separations Methods Technologies Inc.	Deactivated, horzontally polymerized mixed monomeric C_{18}–C_1
E	Phenomenex Ultracarb 5 ODS(20) Phenomenex Inc.	High carbon loaded monomeric C_{18}
F	Zorbax ODS-C_{18} Rockland Technologies Inc./Dupont	Monomeric C_{18}

[a] All columns are 4.6 × 250 mm I.D. × length, With 5µm nominal particle size.

Comparison of these chromatograms indicates that the presence of acids is essential to the complete separation and elution of the seven analytes. As reported in Dalluge et al., the improvement in chromatography is also observed when 0.05% trifluoroacetic acid is replaced with 0.5% acetic or formic acid and therefore represents a pH effect rather than an effect attributed especially to trifluoroacetic acid.

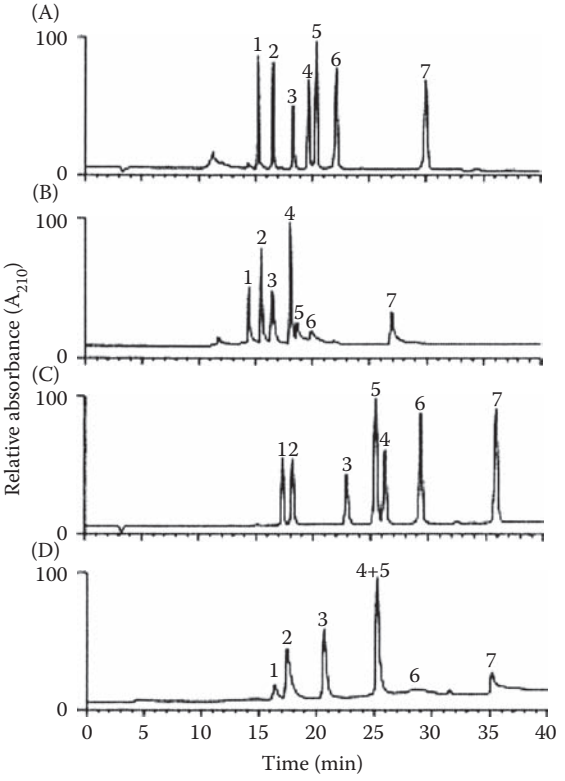


FIGURE 21.3 The effect of acid on the separation of a standard catechin mixture using the deactivated monomeric C_{18} column B (see Figure 21.2). (A) Elution system 1: water + acetonitrile (for details, see (From Dalluge, J.J., Nelson, B.C., Brown Thomas, J., and Sander, L.C., *J. Chromatogr. A*, 793, 265–274, 1998. With permission.) using a mobile phase containing 0.05% trifluoroacetic acid (TFA); (B) elution system 1 using a mobile phase without TFA; (C) elution system 2: water + 0.05% TFA + 60:40 methanol–acetonitrile + 0.05% TFA (for gradient details) using a mobile phase containing 0.05% TFA; (D) elution system 2 using a mobile phase without TFA. Peak identification is the same as in Figure 21.2.

If the main aim of the study is to determine the major flavonoids in a sample, run times of 0.5–1 h usually are enough to separate the 5–10 compounds of interest [78,79]. If a more complete separation of constituents is expected, run times up to 2 h may be required. Under such conditions, even 30–50 flavonoids can be effectively separated and identified in a single run, including such conjugates as glycosides, malonates, acetates, and aglycones [80,81]. As already mentioned, the use of UPLC systems significantly shortens the analysis time [77].

In several publications, instead of linear gradients rather complicated gradient profiles are used, comprising several steps and applying various slopes, without any explanation [1]. Often trial and error plays a large role. Rarely are computer-assisted methods applied for optimization of the gradient profile. Krauze-Baranowska et al. have used the DryLab software for optimization of gradients for separation of 12 biflavones, eight flavonoid glycosides, and three aglycones present in some species from the Ginkgoaceae, Pinaceae, and Podocarpaceae families from the Gymnospermae group [82]. Experiments were performed on a C_{18} column eluted with an aqueous mobile phase adjusted to pH 2.5 with citrate buffer containing an organic modifier (methanol, acetonitrile, tetrahydrofuran).

LC is usually performed at room temperature, but temperatures up to 40°C are sometimes recommended to reduce the analysis time and because thermostated columns give more repeatable elution times.

21.5 DETECTION

21.5.1 UV-VIS Detection

Detection of (iso)flavonoids in food is usually done by UV–VIS with DAD. All flavonoids contain at least one aromatic ring and absorb UV light. The first maximum, located in the range 240–285 nm (band II) and present in all types of flavonoids, is due to the A-ring (see Figure 21.4). The second maximum, in the range 300–550 nm, is due to the substitution pattern and conjugation of the C-ring (band I). Simple substituents such as methyl, methoxy, and nondissociated hydroxyl groups generally effect only minor changes in the position of the absorption maxima [1].

Anthocyanins show band II and band I absorption maxima in the 265–275 and 465–560 nm regions, respectively [62]. Because there is little or no conjugation between the A- and B-rings, the UV spectra of flavanones and isoflavones usually have an intense band II peak but a small band I peak. This lack of conjugation also results in small band I peaks for the catechins (Figure 21.4). The UV spectra of flavones and flavonols have a band II peak at around 240–280 nm and a band I peak around 300–380 nm. As is seen from the sample spectra shown in Figure 21.4 and from the literature reports, the typical wavelengths for analysis and quantification of anthocyanins are 502–525 nm. Catechins are generally quantified at 210 and 280 nm.

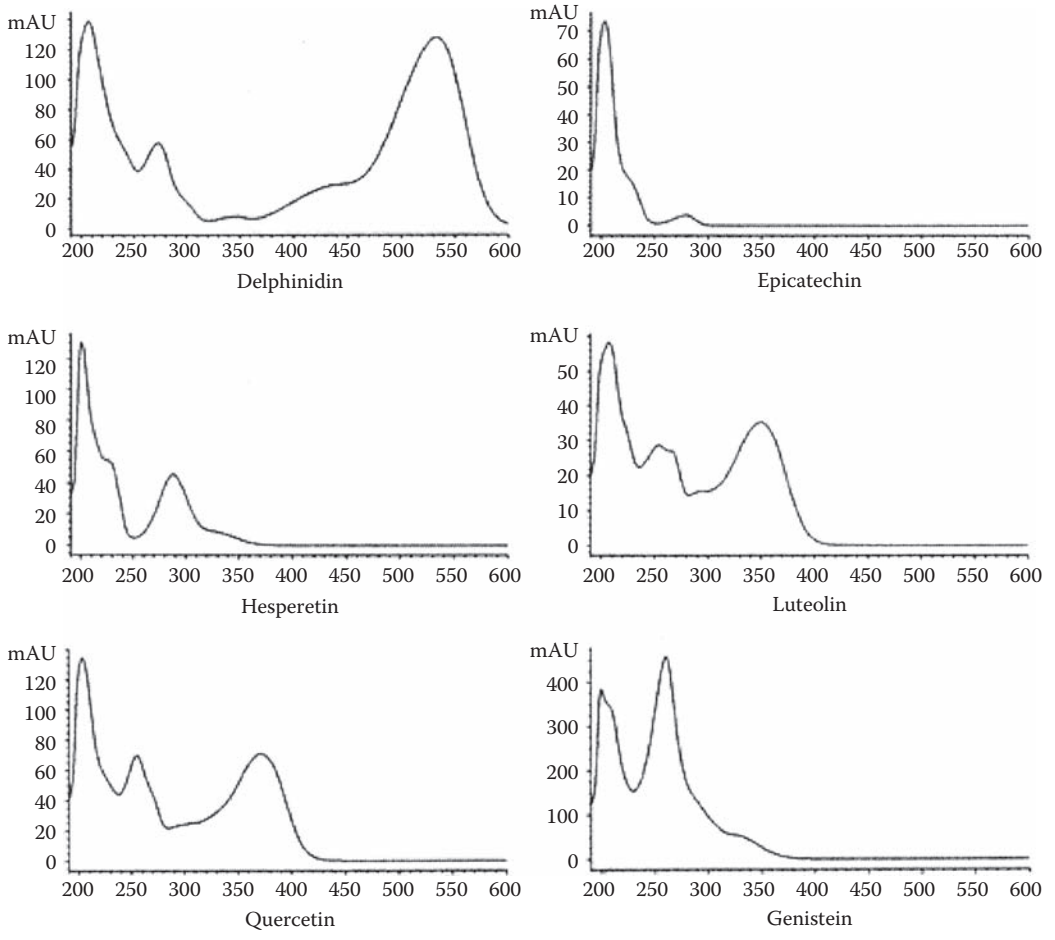


FIGURE 21.4 UV-VIS spectra of an anthocyanidin (delphinidin), a catechin (epicatechin), a flavanone (hesperetin), a flavone (luteolin), a flavonol (quercetin), and an isoflavone (genistein).

Flavanones and their glycosides are generally detected at 280 and 290 nm. Flavones, flavonols, and flavonol glycosides are usually detected at wavelengths such as 270–280 and 350–370 nm. Isoflavones were generally detected at 236, 260, and 280 nm. The spectra shown in Figure 21.4 and the λ_{max} values for particular flavonoids [1] indicate that the various flavonoid subclasses can be distinguished from each other, that is, that LC-DAD is a complementary tool during structural characterization.

Downey and Rochfort report the simultaneous identification of 20 anthocyanins and 9 flavonols in grape skin by HPLC using a C_{18} column and a gradient of methanol–water acidified by formic acid [83]. The use of UV DAD at 520 nm allows for the identification of only anthocyanins, which have their λ_{max} near 520; other flavonoids were not detected at this wavelength (see Figure 21.5a). The same separation monitored at 353 nm allows for the identification of flavonols (Figure 21.5b), though it must be noted that other metabolites also absorb at this wavelength, including many of the anthocyanins. Nine flavonols were identified, all of which were well resolved.

21.5.2 Fluorescence Detection

In flavonoid analysis, fluorescence detection is not used very often [60], because the number of flavonoids that exhibit native fluorescence is limited. Classes of flavonoids that show native fluorescence include the isoflavones, flavonoids with an OH group in the 3-position (e.g., 3-hydroxyflavone), catechins, and methoxylated flavones (e.g., 3′,4′,5′-trimethoxyflavone). As the excitation and emission spectra usually fall in the UV range, this property is very advantageous to the analyst because fluorescence increases sensitivity and minimizes background interference, thus simplifying sample preparation. When fluorescence detection is used in combination with UV, it offers the possibility to discriminate between fluorescent and nonfluorescent coeluting compounds. Figure 21.6 illustrates the selectivity of fluorescence detection for the determination of 3′,4′5′-trimethoxyflavone in an extract of *Flos primulae veris*. Fluorescence detection (LOD (signal-to-noise ratio [S/N] = 3), 25 μg/L) was 10 times more sensitive than UV detection [84] (compare Figures 21.6a and b).

To extend the application range of fluorescence detection, derivatization of nonfluorescent flavonols could also be used. For example, [1] flavonoids with 3-OH and 4-keto substituents can form complexes with metal cations, some of which are highly fluoresecent [85,86]. Postcolumn derivatization of quercetin and kaempferol with Al (III) ions was used for their fluorescence detection [85].

21.5.3 Electrochemical Detection

Recently, electrochemical detection (ED) of phenolics based on their redox properties has gained diffusion [60,87]. Since most flavonoids are electroactive due to the presence of phenolic groups, ED can also be used. Although ED is not as sensitive as fluorescence detection, LODs can be quite low. With the recent advances in ED, multielectrode array detection is becoming a powerful tool, compatible with gradient elution, for detecting flavonoids in a wide range of samples. Determination of the antioxidant activity of phenolic compounds, including flavonoids, by use of RPLC with ED has been performed [88].

21.6 HYPHENATED TECHNIQUES

21.6.1 LC-MS and LC-MS/MS

With the introduction of MS, the coupling of LC and MS has opened the way to extensive, routine analysis of flavonoids [60]. As already mentioned, Figure 21.6 shows determination of 3′,4′,5′-

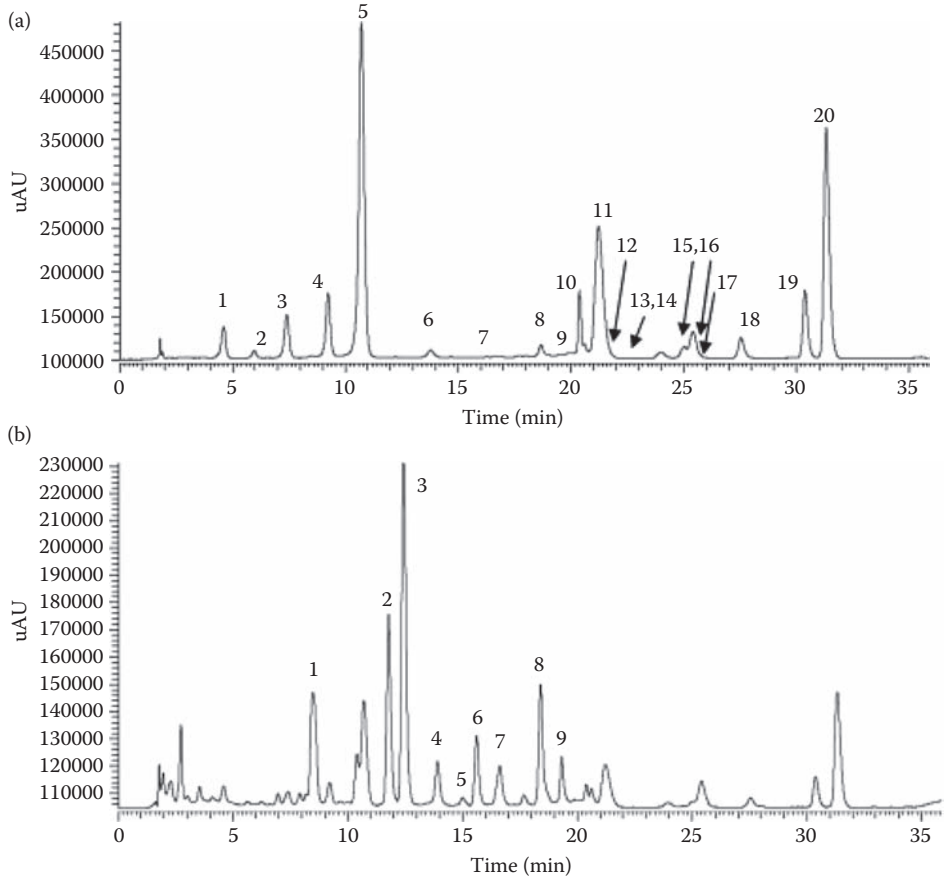


FIGURE 21.5 (a) HPLC chromatogram of Shiraz skin extract at 520 nm. System: C_{18} column, methanol–water gradient containing 10% formic acid. Identified anthocyanins: (1) Delphinidin-3-*O*-glucoside; (2) Cyanidin-3-*O*-glucoside; (3) Petunidin-3-*O*-glucoside; (4) Peonidin-3-*O*-glucoside; (5) Malvidin-3-*O*-glucoside; (6) Delphinidin-3-*O*-acetylglucoside; (7) Cyanidin-3-*O*-acetylglucoside; (8) Petunidin-3-*O*-acetylglucoside; (9) Peonidin-3-*O*-acetylglucoside; (10) Petunidin-(6-*O*-caffeoyl)glucoside; (11) Malvidin-3-*O*-acetylglucoside; (12) Delphinidin-3-(6-*O*-coumaroyl)glucoside (*cis*); (13) Peonidin-(6-*O*-caffeoyl)glucoside; (14) Peonidin-3-(6-*O* -coumaroyl)glucoside (*cis*); (15) Malvidin-3-(6-*O*-coumaroyl) glucoside (*cis*); (16) Malvidin-(6-*O*-caffeoyl)glucoside; (17) Cyanidin-(6-*O*-coumaryoyl)glucoside (*trans*); (18) Petunidin-(6-*O*-coumaryoyl)glucoside (*trans*); (19) Peonidin-3-(6-*O*-coumaroyl)glucoside (*trans*); (20) Malvidin-3-(6-*O*-coumaroyl)glucoside (*trans*). (Downey, M.O. and Rochfort, S., *J. Chromatogr. A*, 1201, 43–47, 2008. With permission.); (b) HPLC chromatogram of Shiraz skin extract at 353 nm. Chromatographic conditions as in Figure 21.5a. Identified flavonols: (1) Myricetin-3-*O*-glucoside; (2) Quercetin-3-*O*-glucuronide; (3) Quercetin-3-*O*-glucoside; (4) Laricitrin-3-*O*-galactoside; (5) Kaempferol-3-*O*-glucoside; (6) Laricitrin-3-*O*-rhamnose-7-*O*-trihydroxycinnamic acid; (7) Kaempferol-3-*O*-caffeoylate; (8) Isorhamnetin-3-*O*-glucoside; (9) Syringetin-3-*O*-galactoside. (From Downey, M.O. and Rochfort, S., *J. Chromatogr. A*, 1201, 43–47, 2008. With permission.)

trimethoxyflavone in an extract of *Flos primulae veris* by various detection modes. With MS in the selected ion monitoring (SIM) mode, detectability was even better (LOD, 5 µg/L) than by use of fluorescence detection (LOD, 25 µg/L) (compare Figure 21.6 b, c, and d) [84].

LC-MS combines the efficient separation capability of LC and great structural characterization power of MS. Compared with GC-MS, LC-MS can determine polar analytes without the need for prior derivatization. This advantage of LC-MS is particularly attractive when simultaneously

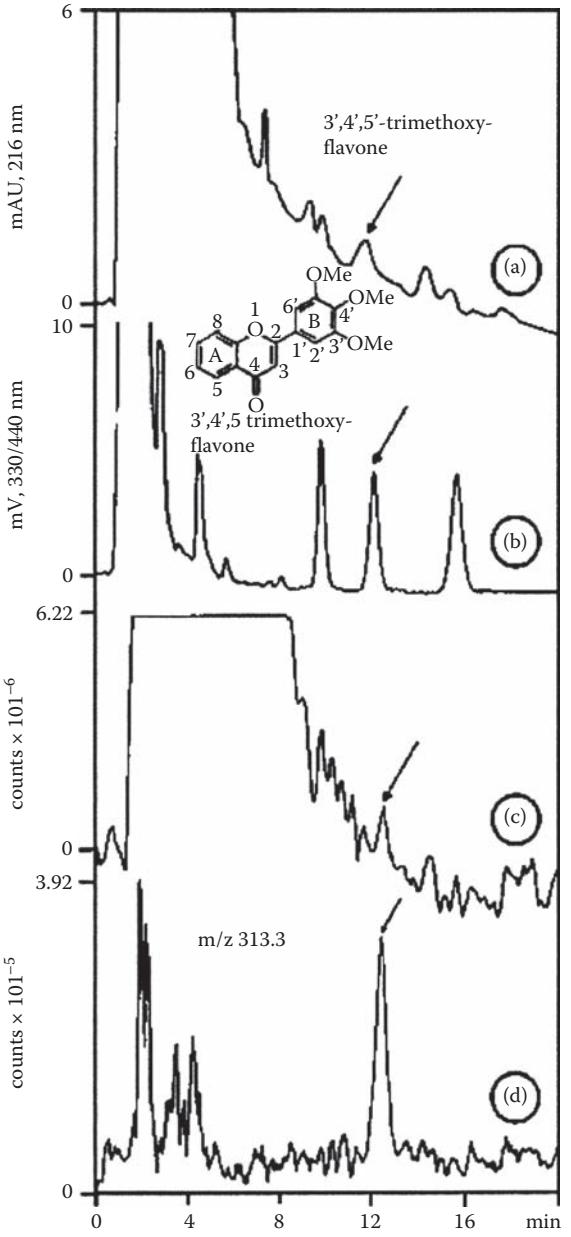


FIGURE 21.6 Comparison of detection methods for 3′,4′,5′-trimethoxyflavone in an extract of *Flos primulaeveris*: (a) reversed-phase liquid chromatography–UV_{216}; (b) reversed-phase liquid chromatography–fluorescence detection (excitation, 330 nm; emission, 440 nm) and reversed-phase liquid chromatography–electrospray ionization (+)–mass spectrometry; in (c) full-scan and (d) extracted ion chromatogram of *m/z* 312–314. (From Huck, C.W. and Bonn, G.K., *Phytochem. Anal.*, 12, 104–109, 2001. With permission.)

analyzing compounds belonging to structurally distinct groups. In most cases single-stage MS is used in combination with UV detection to facilitate the confirmation of the identity of flavonoids in a sample with the help of standards and reference data.

With regard to structural characterization of flavonoids, information can be obtained on (1) the structure of the aglycone, (2) the types of carbohydrates or other substituents present, (3) the

sequence of the glycan part, (4) interglycosidic linkages, and (5) attachment points of the substituents to the aglycone.

In LC-MS of flavonoids, electrospray ionization (ESI) and atmospheric pressure ionization interfaces, that is, APCI, are most often used. These two interfaces show greater ionization stability, and more sensitivity than other interfaces. Both positive ionization (PI) and negative ionization (NI) are applied to yield $[M-H]^+$ and $[M-H]^-$ ions, respectively, although the LOD can be 10–100 times greater than with UV [89]. ESI is particularly appropriate for profiling flavonoids with minimal fragmentation. Thus, coeluting flavonoids with differing masses are readily detected in a three-dimensional chromatogram, where the *m/z* value is the third dimension (see Figure 21.7) [89]. For the identification of unknowns, tandem mass spectrometry (MS/MS or MS*n*) is used—a technique that deserves more attention.

Recently, a new ionization method for LC-MS, atmospheric pressure photoionization (APPI), has been introduced, which is based on charge transfer to the analytes from dopant molecules (e.g., toluene) that have been ionized using 10 eV photons produced by a vacuum-ultraviolet lamp. Matrix-assisted laser desorption ionization (MALDI) is another soft ionization technique; although MALDI–TOF (time-of-flight)–MS is well known as powerful tool for analysis of a wide range of biomolecules, its potential in flavonoid analysis has been explored only recently. Details on fragment ions observed for selected flavonoid classes taken from cited literature are collected in the review article by de Rijke et al. [1].

As an example, the MS and MS/MS spectra of apiginin are presented in Figure 21.8. Loss of hydrogen leads to an (M-H)+ peak. This can be from a hydroxyl (or a methoxyl) hydrogen. The molecular ion [M-H]+ of 271 Da present in the MS spectrum can lose, for example, CO of 28 Da and yield a radical cation of 243 Da, can lose H_2O-CO and form a cation of 225 Da, can lose a C_2H_2O fragment to form a 229 Da cation, and so on; see the MS/MS spectrum.

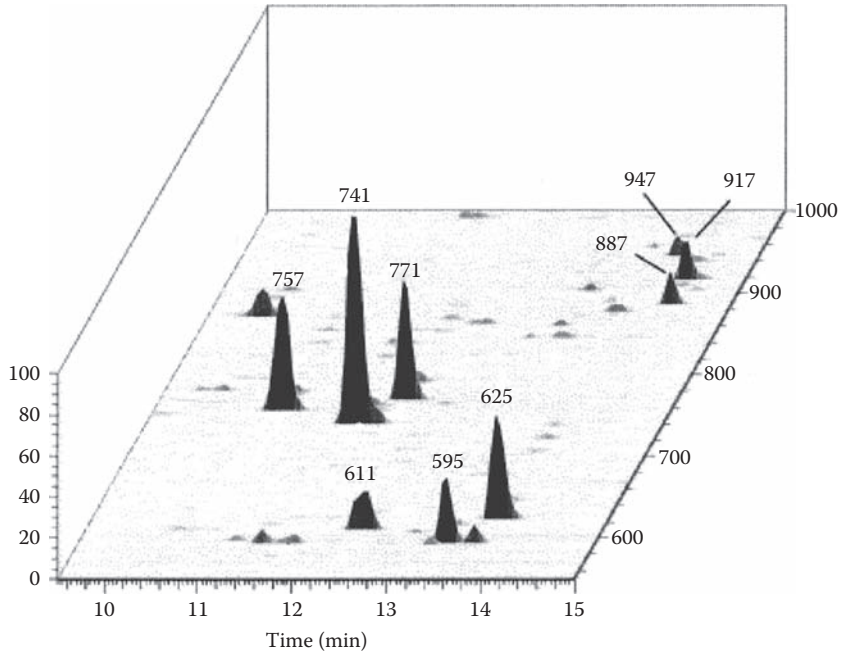


FIGURE 21.7 Three-dimensional plot of a liquid chromatography–electrospray ionization (ES)–mass spectrometry analysis of flavonoids in a crude 80% methanol extract of leaves of Ateleia cubensis. ES protonates flavonoid glycosides with minimal fragmentation, thus coeluting flavonoids with different masses are readily revealed. Numbers above peaks are the m/z values of the $[M + H]^+$ ions of the flavonoids. (Adapted from Kite, G.C., Veitch, N.C., Grayer, R.J., and Simmonds, M.S.J., *Biochem. Syst. Ecol.*, 31, 813–843, 2003. With permission.)

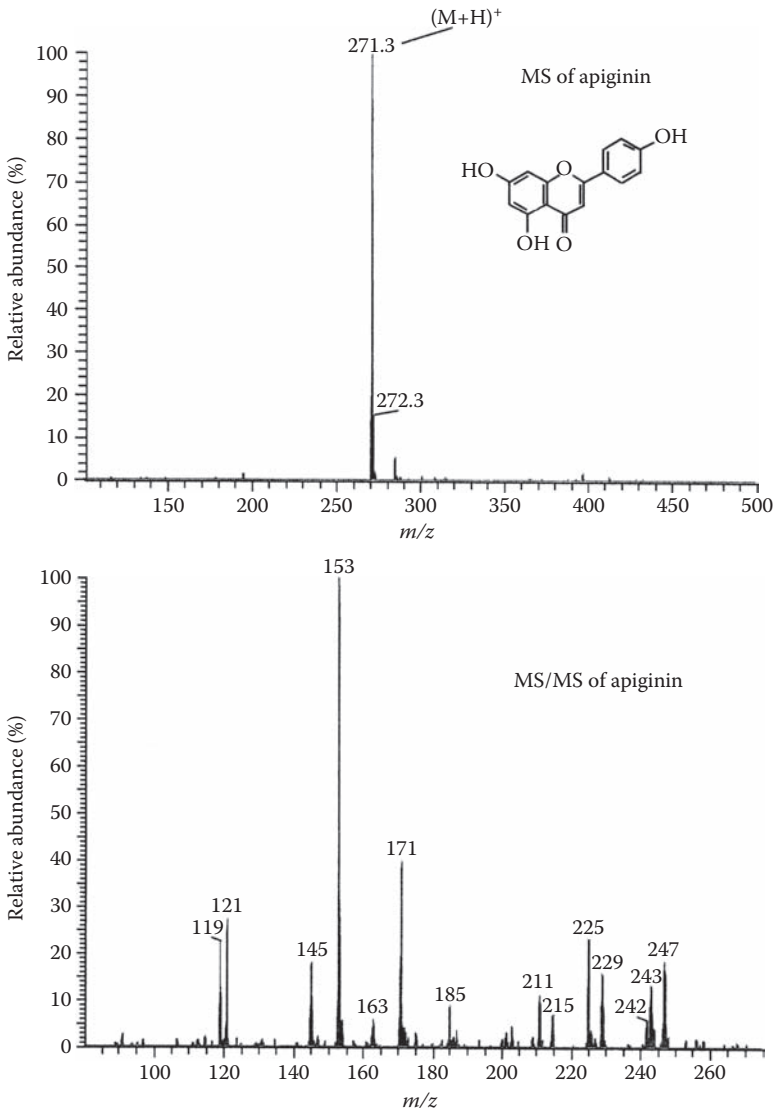


FIGURE 21.8 Structure and full-scan mass spectrum (MS) of apiginin (upper trace) and full-scan tandem mass spectrometry (MS/MS, *m/z* 271) spectrum of apiginin (lower trace). (From www.thermo.com. With permission.)

LC-UV-MS can be used to demonstrate the existence of the same flavonoid glycosides in related taxa, and sufficient spectral data may be provided to assign the structures of common monoglycosides and diglycosides by comparison of the mass and UV spectra and retention times with standards. The complete structures of more complex glycosides can be obtained only by NMR, either off-line, after isolating the compound, or possibly by LC-NMR [89].

21.6.2 LC-NMR

Recently, on-line (often stopped-flow) LC-NMR has attracted increasing attention in the field of natural product research. In numerous papers, the main advantages (differentiation of isomers and substitution patterns) and disadvantages (low sensitivity, long run times, high cost of instruments) have been discussed [1,90,91]. In the case of flavonoids, NMR detection is powerful for

the differentiation of isomers, sugar configurations, and substitution patterns on the aromatic ring system, while MS techniques are needed to obtain information on molecular mass and functional groups. This means that tandem LC-MS-NMR systems have to be used for full structure elucidation [91].

In most publications reporting the use of NMR detection, stopped-flow mode was used to enable very long scan times or very low flow rates to record the NMR spectra [92]. Moreover, for a comprehensive structural elucidation of a novel natural product, preparative isolation is often still necessary [93,94].

21.7 THE APPLICATION OF HPLC OF FLAVONOIDS

Knowledge of the flavonoid content of plant material, plant-based foods, and plant-derived drugs is paramount to understanding their role in plant physiology and human health. In addition, such knowledge has been employed as the basis of chemotaxanomic systems [62] because flavonoids are applied as chemotaxonomic markers. The knowledge of flavonoid content has been extended to the identification of adulteration of plant-derived drugs as well as plant-based foods such as wines, beverages, and so on.

Many papers reporting flavonoid analysis of plants and plant material have been published in journals connected with chromatography, plant chemistry, food chemistry, and pharmaceutics. Recently, a lot of papers deal with a hyphenated LC-MS technique, for example, for identification of flavonoids in wines [95], the genus *Citrus* [96], leaves of *Adinandra nitida* [97], extract of *Sophora flavescens* [98], *Cistus salvifolius* leaves [99], and leaves of *Maytenus ilicifolia* [100].

A very important field of flavonoid analysis is for plants used for medicinal purposes in the form of medicinal herbs or in pharmaceutical preparations. RP-HPLC with UV detection or DAD was used for determination of bioactive flavonoids in rhizome of *Alpinia officinarum* [34], fruits of *Silybum marianum* [101], flos *Inulae* [102], herb of *Artemisia annua* from different sources [103], rhizome of *Smilacis glabrae* [104], *Glinus lotoides* seeds and tablet formulations [105], *Smilax china* [106], inflorescence, leaves, and fruits of *Sorbus* sp. [107], *Ginkgo biloba* phytopharmaceuticals [108], and *Achillea millefolium* herb [109]. Camarda et al. [11] compares the antiproliferative activity and flavonoid composition of *Citrus* juices. The use of HPLC for monitoring the flavonoid content in hairy-root cultures of *Scutellaria baicalensis* was also reported [110].

Optimization of the chromatographic system was performed for screening of numerous medicinal plants and raw cosmetics by Wang and Li [111]. Since the investigated plants consisted of many compounds with different properties, some components being highly polar and some medium polar, the separation was very complicated. Different proportions of organic solvents (methanol or acetonitrile and water mixtures) and gradient elutions in two modes (methanol + phosphate buffer at pH 2 + water in different profiles) in about 30 min were optimized for separation of 12 flavonoids (catechin, rutin, myricetin, fisetin, quercetin, naringenin, hesperetin, luteolin, kaempferol, apigenin, flavone, and flavanone). Procedures have been validated and used for quantitative determination of 10 flavonoids in the following 24 medicinal plants: *Trigonella foenum-graecum*, *Acacia catechu*, *Sophora japonica*, *Glycyrhiza uralensis* (Leguminosae), *Languas galangal*, *Alpinia officinarum* (Zingiberaceae), *Camelia sinensis* (Theaceae), *Gingko biloba* (Gingkoceae), *Malus pumila*, *Prunus persica* (Rosaceae), *Daucus carota* var. *sativa* (Umbelliferae), *Sapindus mukorossi* (Sapindaceae), *Apis cerana* Fabricius (Apidae), *Cinnamonum japonicum* (Lauraceae), *Biota orientalis* (Cupressace), *Nelumbo nucifera* (Nymphaeaceae), *Magnolia liliflorae* (Magnoliaceae), *Equisetum debile* (Equisetaceae), and *Allium cepa* (Liliaceae). The identity of registered peaks was confirmed by the retention times of the peaks of analytes and of individual standards as well as by UV spectra taken from photodiode array detector.

For determination of rutin in plant extracts, a method using a C_{18} column, isocratic elution with acetonitrile–aqueous acetic acid, and UV detection at 252 nm has been elaborated [112]. Flavonoid analysis is also performed to investigate the quality of foods such as wines and grapes [113,114], fruits [115–117], vegetables [118,119], and plant materials [120].

There are reports of the use of RP-HPLC in chemotaxonomic investigations, for example, in identification of *Epilobium* species (Onagraceae) [121] and *Epimedium* species [122] with flavonoids as chemotaxonomic markers. HPLC of flavonoids was also used in botany and plant physiology investigations [123–127].

21.8 HPLC ANALYSIS OF CHIRAL FLAVONOIDS

Within the large family of flavonoids, flavanones present a unique structural feature, known as chirality, which distinguishes them from all other classes of flavonoids [128]. Almost all the flavanones have one chiral carbon atom in position 2, except for a subclass of flavanones named the 3-hydroxyflavanones or dihydroflavonols, which have two chiral carbon atoms in positions 2 and 3. Some flavanones possess an additional D-configured mono- or disaccharide sugar in the C-7 position on ring A. These flavanone 7-*O*-glycosides exist as diastereoisomers or epimers that have the opposite configuration at only one or two or more tetrahedral stereogenic centers present in the respective molecular entities. It has been reported that some chiral flavanones are stereochemically unstable, and often racemization or enantiomerization occurs. For compounds with more than one stereogenic center, there is a process called epimerization. Other classes of flavonoids can also demonstrate chirality in some of their members.

As reported in the review by Yáňez et al. [128], no studies have examined differences in the pharmacokinetics and biological activities of the individual enantiomers of chiral flavanones. However, some isoflavone enantiomers are biosynthesized in red clover, garden pea, and alfalfa [129,130], some of which are stereospecifically converted to chiral products.

There is a need for stereospecific assay methods for the quantitation and isolation of flavonoid enantiomers [128]. The direct chromatographic approach has dominated analysis of chiral flavanones by use of chiral polymer phases of oligosaccharides and their derivatives. Also, chiral mobile-phase additives, such as cyclodextrins, have been applied. Chiral derivatization techniques have been rarely used [128].

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22 HPLC of Lignans

Annika I. Smeds

CONTENTS

Of all chromatographic techniques, high performance liquid chromatography (HPLC) seems to be the most commonly used for the separation of lignans today, at least judging by the number of publications in international scientific journals. The introduction of advanced hyphenated techniques such as liquid chromatography (LC)–electrospray ionization (ESI)–mass spectrometry (MS), enabling quantification of lignans at low levels in complex matrices in a rapid and reliable way, has caused a rapid growth during the last 5–10 years of lignan analysis by LC-based techniques. Appropriate analytical techniques are required for lignans because of their large significance, with potential applications in medicine and nutrition. This chapter focuses on sample-preparation procedures and on different methods for lignan analysis by LC techniques. Because of the limited length of this chapter, only a selected part of the huge number of studies on lignans overall, and HPLC lignan analysis particularly, is presented here.

22.1 INTRODUCTION

Lignans are a group of phenolic compounds found throughout the plant kingdom. They were identified in trees already in the 1890s [1,2], and the name *lignan* was proposed by Haworth in 1936 [3]. Until now, nearly 500 different lignans have been identified in plants [4]. Lignans occur widely in vascular plants—they have been found in, for example, stems, roots, leaves, fruits, and seeds. Due to the widespread occurrence of lignans in plants and in the diet of humans and animals, they have recently been shown to be widespread in the aquatic environment as well [5,6] and in foods of animal origin [7]. In trees, they occur in their free form (as aglycones), whereas in other plants they occur at least partly in the conjugated form.

Structurally, lignans consist of two phenylpropane units linked together with β,β-bonds. In some plants from the Magnoliale and Piperale family, some lignans have been identified that are linked together with other carbon–carbon bonds; these are called neolignans [4]. In trees, trimeric (sesquilignans) and tetrameric lignans (dilignans) occur, representing a group called oligolignans [8]. The molecular structures of some lignans representing different structural groups are shown in Figure 22.1.

The biological functions of plant lignans are largely unknown. Lignans have been shown to induce a wide variety of biological effects, including antioxidant, antitumor, antifungal, insecticidal, and antiviral activities [4,9,10]. Derivatives of the lignan podophyllotoxin, present in *Podophyllum* plants, are used as drugs in cancer treatment [4]. The antifungal and insecticidal effects of several lignans suggest that they may play a role in plants' chemical defense against external stress factors. This is especially important for trees because of their long life span, and they indeed contain a high amount and a wide variety of lignans and other bioactive phenolic substances [8,11–14]. Lignans are concentrated in the knots of trees, that is, the branch roots inside the stem. The richest known natural lignan source is knots of Norway spruce (*Picea abies*), which may contain up to 30% (w/w) lignans [11], with 7-hydroxymatairesinol (HMR) as the predominant lignan.

In foodstuffs, secoisolariciresinol (Sec) and matairesinol (MR) were the first lignans identified [15]. Later, other lignans, that is, lariciresinol (Lar), pinoresinol (Pin) [16], syringaresinol (Syr), and medioresinol (Med), were also found to occur commonly in foodstuffs [17,18], and the list was recently further extended with 15 lignans [18].

Most of the dietary lignans are metabolized by gut microbiota, mainly to enterolignans such as enterolactone (EL) and enterodiol (ED). These enterolignans are usually present in the body fluids of humans and mammals; however, they have recently been shown to be widespread also in the aquatic environment [5,6] and even in plants, due to the roots' uptake of enterolignan-containing water [6].

22.2 SAMPLE-PREPARATION PROCEDURES

22.2.1 Sample Handling of Plant and Food Materials

The sampling procedure, storage, and pretreatment of samples are very important issues for the outcome of lignan analysis. These procedures may involve several workup steps with risks for contamination, loss of sample, and unexpected reactions of the lignans [19]. The samples should preferably be placed in a freezer immediately after sampling, to avoid unwanted reactions such as oxidation or polymerization of sensitive compounds. A mild drying technique for the plant material should be chosen, such as freeze-drying or, if unavailable, drying at room temperature. Grinding may also cause unwanted reactions due to generation of heat in the mill. The ground or pulverized samples should be stored in a cold, dark, and dry place.

22.2.2 Extraction Methods

The traditional method for extracting lignans from plants is Soxhlet extraction or percolation with a polar solvent. It is advisable to start by removing lipophilic compounds using a nonpolar organic solvent, because they may disturb the subsequent lignan analysis [8,9,20]. The more hydrophilic extractives, including the lignans, can then be extracted using a polar solvent, such as acetone or methanol. Adding 5–30% water to the solvent increases the penetration ability of the solvent and promotes the extraction of more polar compounds such as lignan glycosides.

Accelerated solvent extraction (ASE) is an excellent method for extraction of lignans in plant samples. It is performed at an elevated temperature and pressure and under an inert nitrogen atmosphere [11,12]. This method enables rapid, automated, and sequential extraction, using relatively small solvent volumes. It has been applied for extraction of lignans from different tree species [11–14] and recently also from cereal, nut, and oilseed species [6,18].

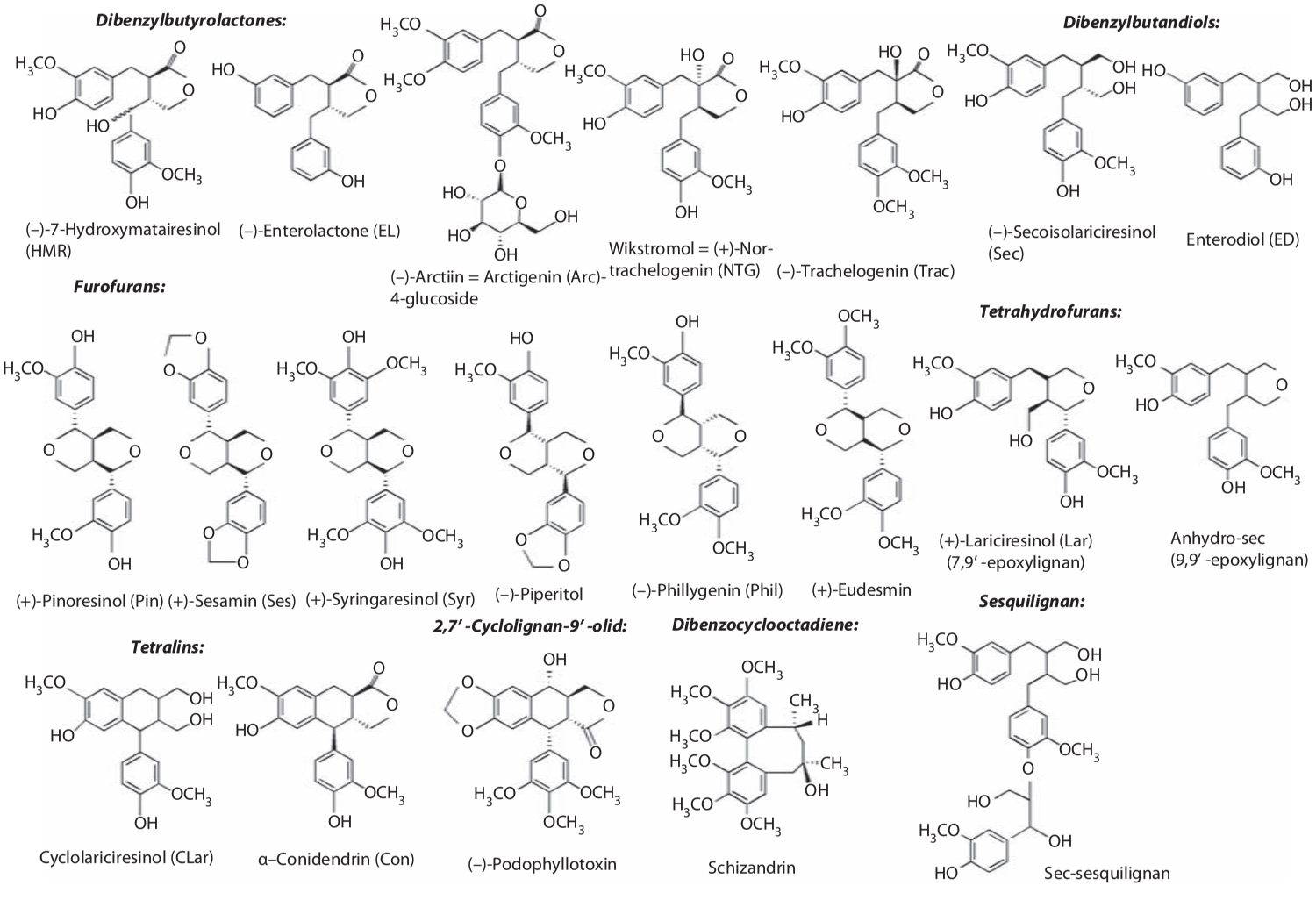

FIGURE 22.1 Molecular structures of some lignans representing different structural groups.

In plants (except in tree species) and plant foods, lignans occur partly in an esterified form bound to the matrix and/or glycosidically linked to carbohydrates. Sec diglucoside (SDG) in flaxseed is an example of such lignans. SDG has been shown to be present in the plant as a macromolecule, that is, mainly as ester-linked oligomers consisting of SDG and 3-hydroxy-3-methylglutaric acid [21,22]. After alkaline hydrolysis or methanolysis, which as such are used as the extraction step by using elevated temperatures and stirring, shaking, or sonication, an additional enzymatic hydrolysis step using either β-glucuronidase, β-glucosidase, or cellulase is usually performed in order to simplify the subsequent chromatographic analysis [17,18,23–25]. Alternatively, strong acid hydrolysis may be applied in order to break both ester linkages and glycosidic bonds [26,27]; however, this treatment degrades acid-labile lignans (see Section 22.2.3).

In biological fluids, the major part of the lignans are conjugated as glucuronides and sulfates, and therefore these samples are usually enzymatically hydrolyzed using β-glucuronidase/sulfatase preparations prior to sample extraction, which often is performed using solid-phase extraction (SPE) [25,28–33]. SPE has also been applied in the analysis of lignans in flaxseed and in the analysis of sesamolinol diglucoside in sesame seeds, where it has been used as a purification step or for fractionation of SDG oligomers in a raw extract [22,34,35]. Liquid–liquid extraction with ethyl acetate has been used for isolation of lignans after enzymatic hydrolysis of food sample extracts [17,18,24], or with diethyl ether after enzymatic hydrolysis of biological fluids, with a subsequent purification step using ion-exchange chromatography [36–38].

Supercritical fluid extraction (with carbon dioxide) has been used very little in lignan analysis. It seems to work satisfactorily for lignans of low to medium polarity, such as those obtained from plant parts of *Schizandra chinensis* [39,40]. Addition of methanol to the carbon dioxide may raise the extraction efficiency of certain lignans considerably [41].

22.2.3 Artifacts and Unwanted Chemical Transformations during Sample Pretreatment

Artifacts and unwanted chemical reactions such as oxidation, thermal degradation, or polymerization of sensitive lignans are common in lignan analysis and can be avoided by taking simple measures, such as use of an inert atmosphere, absence of light, and avoidance of high temperatures.

One of the most studied lignans, HMR, is very unstable both in strong alkaline and strong acid solutions. Strong acid conditions lead to the formation of α-conidendrin (Con) from HMR [42,43], and under alkaline conditions, HMR also forms α-Con, which in turn is converted to α-Con acid [43–45] and other compounds [43,46]. The two isomers of HMR, which differ in the stereochemistry at C-7, can equilibrate, and both are oxidized to 7-oxo-MR, and further to colored oligomers when exposed to irradiation by light [47] or by auto-oxidization in nonfresh wood samples [20]. Acid conditions can also cause artifacts such as anhydro-Sec and anhydro-cyclolariciresinol (CLar) by elimination of a water molecule from the diol structure, as was shown for the guaiacylglyceryl ethers of these lignans [8]. Anhydro-Sec is also an artifact produced upon acid hydrolysis of Sec or SDG [26]. Sec can be partially transformed to Sec-acetonide during column chromatography with acetone on slightly acidic silica columns [8]. The acetonides of Sec, CLar, and isotaxiresinol have been reported upon isolation of phenolic compounds from *Taxus mairei* [48]. 7-Hydroxy-Sec, reported to occur naturally in some trees [14] and foodstuffs [18], can be transformed to Lar and CLar by an acid-catalyzed intramolecular cyclization reaction [46]. Lar will also further rearrange to CLar in acid conditions [31,46,49].

The presence of free radicals in a lignan-containing solution may cause unwanted chemical transformations, as several lignans are known to be effective radical scavengers [9,50]. Not only may lignans form adducts, but dimerization may also occur. Lignan dimers and other adducts may be totally unnoticed during the analysis, thus giving wrong results during quantification.

22.3 METHODS FOR HPLC ANALYSIS

Most HPLC analyses are performed on columns packed with reversed-phase achiral material, as most of the studied lignans are of medium polarity, although silica (normal phase) is sometimes used. Lignans are chiral plant metabolites that mostly occur in an enantiomerically pure form or in enantiomeric excess in plants. The enantiomeric composition in a mixture can be determined by separation on a chiral HPLC column.

In more recent years, especially after the development of LC-MS techniques, UV detection is often used only as a complement to other detection techniques. Usually, LC-MS analyses are performed in the negative-ionization mode, as the acidic phenol groups occurring in most lignans are easily deprotonated. The first ionization technique for LC-MS was thermospray, which was soon replaced by ESI or atmospheric-pressure chemical ionization (APCI). ESI seems to be the preferred ionization technique in HPLC-MS lignan analysis today.

22.3.1 Normal-Phase Separations

For analytical purposes, normal-phase columns have been used mainly for lipophilic lignans, such as lignans present in *Podophyllum* species [51–53] or in sesame seed oils [54]. For most lignans, which are more hydrophilic, normal-phase columns are generally used only for isolation or purification at a preparative or semipreparative scale. Three lignans in *Podophyllum* resin, specifically, podophyllotoxin and α- and β-peltatin, were separated using 1.8% ethanol in chloroform as the mobile phase [51]. Using *n*-heptane–dichloromethane–methanol (90:10:4) as the mobile phase, seven diastereoisomers of podophyllotoxin were successfully separated [52]. Furthermore, a method was developed for quantification of podophyllotoxin in different varieties of *Podophyllum* resins using a mobile phase consisting of *n*-hexane–methanol–tetrahydrofuran–acetic acid (85:10:4:1) [53]. Lignans from *Podophyllum* species can also be successfully separated using reversed-phase (RP) columns. However, for separation of the lipophilic lignans sesamolin and sesangolin in sesame seed oils, a normal-phase column (mobile phase: 6% diethyl ether in *n*-heptane) was found to be superior to a RP column [54].

22.3.2 Normal-Phase Chiral Separations

The normal-phase columns Chiralcel OC [adsorbent: cellulose tris(phenyl carbamate)] and Chiralcel OD [adsorbent: cellulose tris(3,5-dimethylphenyl carbamate)] have been applied in numerous studies for the separation of lignan enantiomers. Mostly, columns of 250 mm length and 4.6 mm ID are used, and they are usually eluted using isocratic flow. Chiralcel OC columns with a mixture of ethanol and hexane as the mobile phase have been used for separation of wikstromol enantiomers isolated from *Wikstroemia sikokiana* [55] and for separation of Lar enantiomers in cell-free extracts of seeds and petioles of *Arctium lappa* L. [56]. Chiralcel OD columns have been used for separation of MR and Pin enantiomers isolated from *W. sikokiana* [55] and of Pin and piperitol enantiomers in sesame seeds [57,58]. As the mobile phase, ethanol–*n*-hexane–acetic acid (15:84:1) was used for MR, and ethanol [55] or ethanol–*n*-hexane (1:1) [57,58] was used for Pin. For piperitols, the mobile phase consisted of ethanol–*n*-hexane (1:4) [57] or (1:9) [58]. Pin and Sec enantiomers in cell-free extracts from seeds and petiols of *Arctium lappa* L. were separated using a mobile phase of ethanol for Pin and ethanol–*n*-hexane–acetic acid (30:69:1) for Sec [56]. Pin enantiomers have also been separated using a smaller Chiralcel OD column (3.5 × 50 mm), which was eluted with ethanol–*n*-hexane (1:1), using UV detection at 280 nm and radiochemical monitoring or inline laser polarimetry [59]. Enantiomers of Sec, anhydro-Sec, Lar, and MR isolated from flaxseed were separated using a mobile phase of ethanol and *n*-hexane [49]. Individual solvent ratios were used for each lignan (65–85% *n*-hexane).

Semi-micro columns of 1.0–2.0 mm ID were found to provide 5- to 20-fold better sensitivity than conventional analytical columns of 4.6 mm ID in chiral lignan analysis [60]. Enantiomers of Pin, Sec, MR, and Lar were separated using Chiralcel OC, OD, or OD-H columns of 1.0, 2.0, and 4.6 mm ID, with UV detection at 280 nm. The mobile phases used consisted basically of ethanol and *n*-hexane. Analyses were also performed using an HPLC-APCI-MS system.

22.3.3 Reversed-Phase Separations

With RP columns, gradient elution is usually applied, with a slightly acidic mobile phase because of the acidity of the phenolic groups. Methanol or acetonitrile have been extensively used as organic solvents in the mobile phase. For the separation of mixtures containing both diastereomers and functional-group derivatives, for example, mixtures of methanol and acetonitrile or dimethyl sulphoxide are required [52]. The most widely used RP column is RP-18 (octadecylsilica); however, RP-8 columns are more suitable for separation of more hydrophilic lignans such as HMR isomers or HMR acid and Con acids [32,43,61].

A standard solution containing 28 pure lignans was analyzed using HPLC–ion trap MS [62]. The separation was performed on an RP-8 column (2.1 × 150 mm, particle size 3.5 μm). The analysis time was 50 min. Several lignans overlapped, but they could be separated by their different mass fragmentation. Very recently, a newer type of LC column, a rapid resolution RP-18 column (2.1 × 100 mm, particle size 1.8 μm), was applied in LC–ion trap MS analysis of a standard solution containing 18 pure lignans. With an analysis time of 25 min, a complete resolution of all lignans was achieved (Figure 22.2) [63].

22.3.3.1 Analysis of Medicinal Herbs

UV detection may offer sufficient selectivity and sensitivity for determination of lignans in plant extracts. In one work, six major lignans in extracts of *Schizandra chinensis* (schisandrins and gomisins) were quantified by HPLC-UV at 254 nm [64]. Eight furofurano lignans (e.g., sesamin

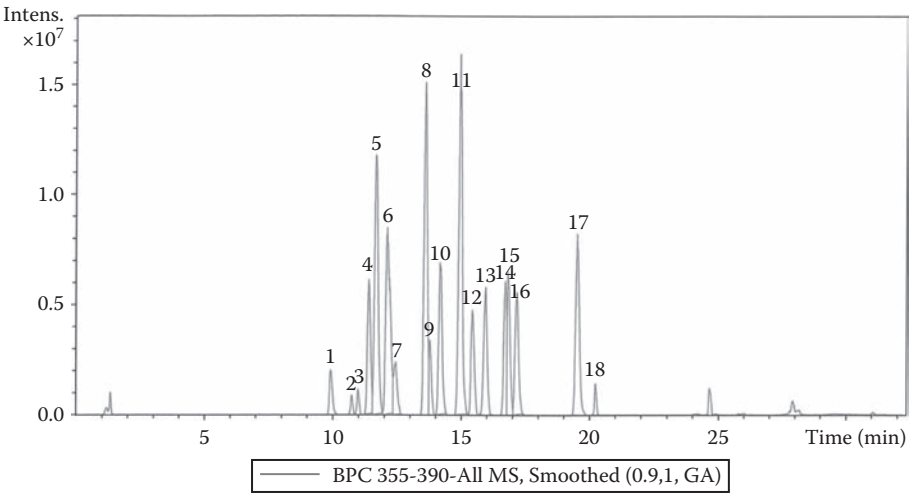

FIGURE 22.2 Rapid resolution liquid chromatography–ion trap mass spectrometry base peak chromatogram of a standard solution containing 18 pure reference lignans. Column C_{18}, 2.1 × 100 mm, 1.8 μm particle size. Eluent A, 0.1% acetic acid; eluent B, methanol–acetonitrile (50:50). Gradient elution from 10 to 30% B in 6 min, then to 50% B in 10 min, then to 80% B in 9 min. Total analysis time 25 min. Peak identification (number): (1) 7-Hydroxy-Sec; (2) Con acid; (3) CLar isomer1; (4) HMR; (5) CLar isomer2; (6) iso-HMR isomer1; (7) iso-HMR isomer2; (8) Sec; (9) Lar; (10) Nortrachelogenin (NTG); (11) 7-oxo-MR; (12) Con; (13) Pin; (14) MR-d_6; (15) MR; (16) Trachelogenin (Trac); (17) Arctigenin (Arc); (18) Phillygenin (Phil).

[Ses], syringaresinol [Syr], epi-Ses, and Phil) were separated on an RP-18 column using UV detection at 240 nm [65]. A method for quantification of epi-Ses and Ses isolated from the roots of *Asarum* species was then developed using an improved chromatographic method, that is, altering the flow rate in an isocratic elution. An advanced method, that is, HPLC-UV at 280 nm coupled to laser polarimetric detection, which includes separation of enantiomeric forms, was developed for identification of lignans and lignan glucosides in crude methanolic extracts of *Forsythia intermedia* [59]. The lignans analyzed were, for example, Pin, MR, Arc, arctiin, Phil, and phillyrin.

In one of the early HPLC-MS applications for lignans, HPLC–thermospray MS coupled with or in combination with UV detection was used for analysis of nordihydroguaiaretic acid in chaparral (*Larrea tridentata*) [66]. This technique was also used for analysis of some lipophilic lignans, that is, eudesmin, magnolin, yangambin, and kobusin in *Polygala* species [67] and Phil, eudesmin, and epieudesmin in leaves of *Orophea enneandra* [68]. HPLC–ESI–single-quadrupole MS coupled to or in combination with a UV detector was used for the identification of 15 lignans (schisandrins and gomisins) and the tentative identification of nine other lignans in extracts of *Schizandra chinensis* fruits [69] and furthermore for the identification of arctiin and Arc in leaves of burdock (*Arctium lappa* L.) [70]. Very recently, the same technique was applied for development and validation of a quantification method for all 15 lignans in extracts of *Schizandra chinensis* fruits [71].

HPLC–quadrupole-MS, although a selective and sensitive method, does not permit characterization of the chemical structure and is therefore suitable mainly for quantification of already known compounds available as pure reference standards. With LC–ion trap MS, which enables multiple fragmentation of selected masses, the possibilities for identification of unknown compounds are better. LC–ESI–ion trap MS was used for deducing the structure of products formed in alkaline solutions of HMR, that is, iso-HMR, HMR acid, and Con acids [43]. HPLC–APCI–ion trap MS was applied for identification of several lignans and lignan glucosides in a crude methanolic extract of *Forsythia intermedia* [59]. Recently, HPLC–ESI–ion trap MS was used for identification or tentative characterisation of 21 lignans in the fruits of *Forsythia suspensa* [72].

Another advanced method developed in recent years, allowing complete structural characterization of compounds in complex mixtures at microgram levels, is HPLC coupled to ^{1}H-nuclear magnetic resonance (NMR) spectroscopy. The structures of Phil, eudesmin, and epieudesmin in *Orophea enneandra* leaves [68], and of seven lignans in *Torreya jackii* needles (e.g., Trac, NTG, Arc, and Pin), were preliminary assigned using this technique [73]. On-line HPLC-NMR-MS was applied for the separation and characterization of two diastereomers of SDG from flaxseed [74]. HPLC-NMR can be further coupled to other techniques: An MS may be coupled to HPLC in parallel with the NMR instrument, or SPE cartridges may be installed between the HPLC column and the detector. This may solve problems with interference from the eluted solvent, which often prevents proper structural elucidation. The structures of nine lignans present in *Phyllanthus urinaria*, specifically, virgatusin, three diarylbutanes, and five aryltetralins, were elucidated using HPLC-SPE-NMR [75].

22.3.3.2 Analysis of Foodstuffs

Examples of lignan-rich foods are oilseeds, especially flaxseed and sesame seeds [15–18]. Legumes, fruits, berries, some other vegetables, and foods of animal origin contain small or moderate amounts of lignans [7,15,16].

Pinoresinols in olive oil were analyzed using HPLC-UV at 278 nm with separation on an RP-18 column [76,77]. Flaxseed lignans, that is, SDG, anhydro-Sec, CLar, Lar, Pin, and MR, have been determined using HPLC with diode array detection (DAD) [34,49,78], which was also used for analysis of lignans in pumpkin seeds [49]. Lignans in a crude extract of unroasted defatted black sesame seeds (Ses, sesamolin, sesamol, sesaminol, and sesaminol di- and triglucosides) were quantified using HPLC-UV at 280 nm [79].

HPLC with coulometric electrode array detection (CEAD) is a technique based on multiple electrochemical detectors in series, maintained at different potentials. This technique was applied for

quantification of lignans in wines [80] and in some plant foods [25]. Also, fluorescence and electrochemical detectors may be useful in HPLC analysis of lignans. In one work, HPLC was used in combination with radiochemical detection for the study of the metabolism of furanofuran lignans (Pin, piperitol, Ses, sesamolinol, and sesamolin) in sesame seeds [57]. Fluorescence detection was found to be superior to UV or electrochemical detection in the analysis of Pin and 1-acetoxy-Pin in olive oil extracts [81].

HPLC-MS/MS has been applied in numerous newer analyses of foodstuff lignans. Two isomers of SDG in a methanolyzed extract of flaxseed meal were identified by HPLC-ESI-MS/MS and by HPLC coupled to continuous flow-fast atom bombardment-MS [82]. HPLC coupled to a heated nebulizer (HN)–APCI–MS/MS in multiple reaction monitoring (MRM) mode was used for the quantification of Sec and MR in 112 food items [23]. Later, this technique was developed and validated for quantification of Sec, MR, Lar, and Pin in different foods [24]. HPLC-ESI-MS/MS (MRM) was used for quantification of 24 plant lignans in sesame seeds and flaxseed, 16 cereal species, and four nut species [18]. Recently, HPLC coupled to a combined triple-quadrupole-ion trap system has also been used for quantification of lignans in foodstuffs [7,83].

22.3.3.3 Analysis of Biological Fluids

HPLC-UV has had a limited use in the determination of lignans in biological samples, because of its insufficient sensitivity and selectivity. This method has been used for the quantification of Pin and Lar in extracts of bacteria and general anaerobic-medium broth mixtures (at 280 nm) [84]. HPLC-UV (at 283 nm) and -DAD were used for the analysis of Sec and MR metabolites in extracts of Sec or MR incubations with rat or human liver microsomes [85].

Examples of sensitive and selective methods suitable for quantification of lignans in biological samples are HPLC with CEA or MS detection, especially HPLC-MS/MS (MRM). The HPLC-CEAD method is limited to lignans with free phenolic hydroxyl groups, whereas HPLC-MS is limited to compounds with ionizable groups, although this is usually not a problem with lignans. HPLC-CEAD has been used for the quantification of ED and EL in blood plasma and uterine tissue of rats [86] and in human urine samples [86,87]. The technique was also applied for validating a method for quantification of eight lignans in human urine [37]. Furthermore, HPLC-CEAD methods were validated for the quantification of several lignans in human plasma [36,38]. Urinary levels of ED and EL in female subjects were measured using HPLC-HN-APCI-MS/MS (MRM) [28]. This study seems to have been the first in which lignans have been quantified in urine or blood samples using the HPLC-MS technique. Since then, the technique has been applied in several studies for developing and validating fast, sensitive, and selective methods for quantification of lignans in urine and blood samples. In one study, HPLC-HN-APCI-MS/MS (MRM) was applied for validation of a method for quantification of MR, ED, and EL in human serum and urine [29]. The detection limits were comparable to those achieved with HPLC-CEAD. The analogous technique, but with ESI, probably affords the lowest detection limits of lignans. This technique was applied for quantification of ED and EL in human serum [88] and human plasma [87] and for quantification of six enterolignans and 11 plant lignans in human plasma [30,33]. A large number of lignans were analyzed also in rat urine using this technique [31,32]. A method has been developed for direct quantification of ED and EL and their glucuronides in human serum and urine by HPLC-ESI-MS, without sample pretreatment [89]. Recently, ED and EL were quantified in biological samples using HPLC-ESI coupled to linear ion trap MS [90].

22.3.4 Reversed-Phase Chiral Separations

A Chiralcel OD-R column (250 × 4.6 mm) coupled to ESI-MS/MS was used for the separation of EL enantiomers in rat urine extracts [91]. The mobile phase consisted of methanol–0.1% acetic acid (70:30). EL enantiomers were also separated using a chiral CD-Ph column (250 × 4.6 mm) using acetonitrile–water (33:77) as the mobile phase and UV detection at 280 nm [92].

ACKNOWLEDGMENTS

This work is part of the activities at the Åbo Akademi Process Chemistry Centre within the Finnish Centre of Excellence Programme (2000–2005 and 2006–2011) by the Academy of Finland. Academy Research Fellow Stefan Willför is acknowledged for suggesting the task of writing this chapter, as well as for assistance with some sections of this chapter together with Prof. Bjarne Holmbom.

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Secondary Metabolites — Isoprenoids

23 HPLC of Mono- and Sesquiterpenes

Angelika Koch, Simla Basar, and Rita Richter

CONTENTS

23.1 DEFINITION AND CHEMICAL CLASSIFICATION OF MONO- AND SESQUITERPENES

Terpenes are derived biosynthetically from units of isoprene, which has the molecular formula C_5H_8. The in vivo precursors are isopentenyl pyrophosphate and its isomeric dimethylallyl pyrophosphate. In biosynthesis the isoprene units are linked together to build up the skeletons of the terpenes as a multiple of $(C_5H_8)_n$. (The isoprene rule of Wallach [1887] states that schematic opening of the cyclic skeleton reveals acyclic skeletons that are formally dissected into isoprene units [1].) The isoprene units may be linked together "head to tail" or "tail to tail" (Figure 23.1).

Although differing in their chemical constitution, mono- and sesquiterpenes have many physical properties in common. The boiling points range from 140–180°C (monoterpenes) to 240°C (sesquiterpenes). Other physical characteristics are densities from 0.759 to 1.096 and high refractive indices; most are optically active, and specific rotation is often a valuable diagnostic property mixtures of many constituents.

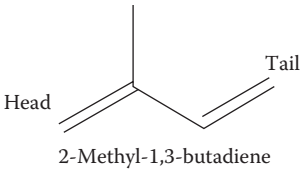


FIGURE 23.1 The isoprene unit.

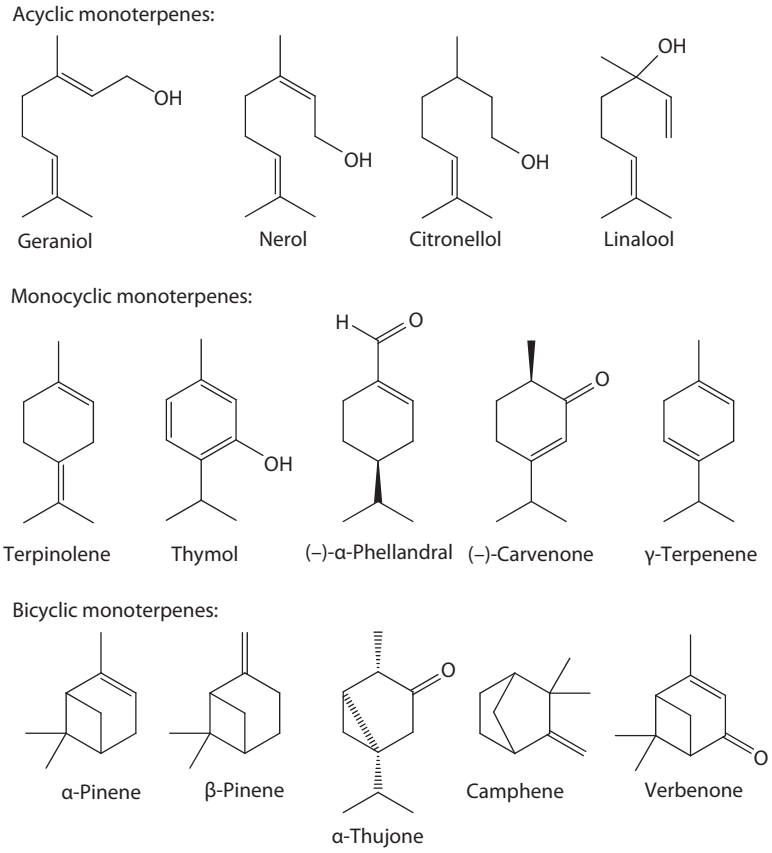


FIGURE 23.2 Classification of monoterpenes.

Monoterpenes consist of two isoprene units and have the molecular formula $C_{10}H_{16}$. Addition of a further C_5 (isoprene) unit forms the C_{15} sesquiterpenes. Adding more of these C_5 units yields the diterpenes (C_{20}), sesterpenes (C_{25}), triterpenes (C_{30}), tetraterpenes (C_{40}), and polyterpenes ($C_{>40}$). With regard to the monoterpenes, these substances can be further divided into three groups depending on whether they are acyclic, monocyclic, or bicyclic (Figure 23.2). Within each group, the monoterpenes may be unsaturated or have functional groups and be alcohols, aldehydes, or ketones. The majority of natural terpenoids have cyclic structures with or without functional groups, sometimes with an aromatic structure (thymol). Also included among the monoterpenes for biosynthetic reasons are the monoterpene lactones, known as iridoids. Like the monoterpenes, the sesquiterpenes are grouped according to their basic carbon skeleton. The common ones are either acyclic, monocyclic, or bicyclic (Figure 23.3).

Some of the volatile compounds are components of the essential oils and serve as starting material for fragrances and pharmaceuticals as well. Isomerism is common among terpenoids, and the

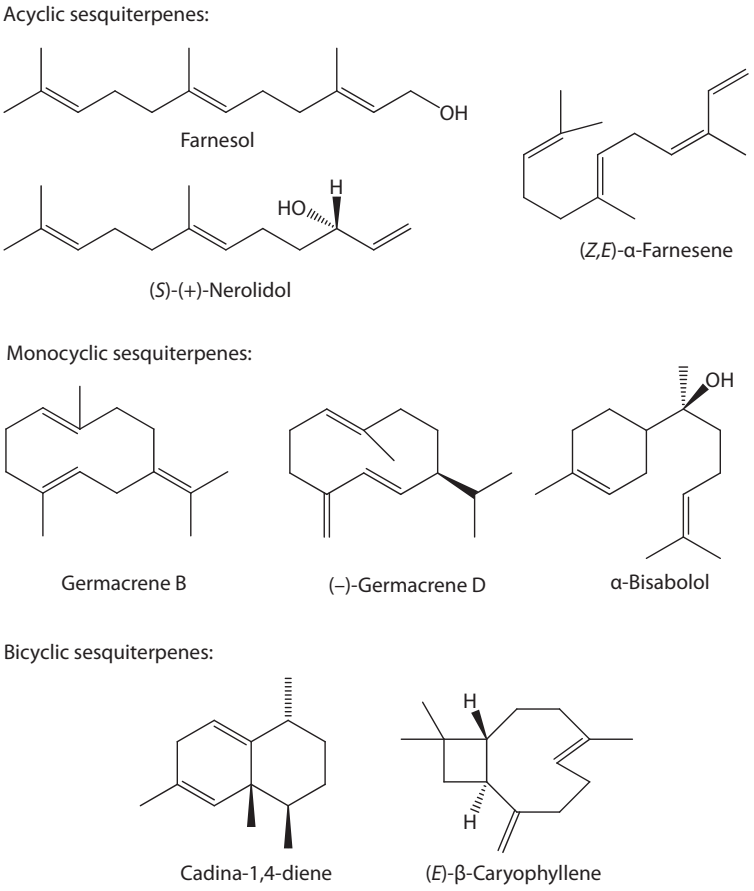


FIGURE 23.3 Classification of sesquiterpenes.

stereochemistry is often difficult to determine. Isomerization and structural rearrangement within the molecule occur quite readily, even under relatively mild conditions. That is why artifact formation is always possible during isolation procedures.

23.2 OCCURRENCE AND IMPORTANCE OF VOLATILE COMPOUNDS IN THE PLANT KINGDOM

Chemically, according to their structure, terpenes are generally lipid-soluble. They are located in the cytoplasm of the plant cell. The volatile essential oils sometimes occur in special glandular cells located at the surface of leaves, flowers, fruits, and seeds. The terpenes of the resins (when dissolved in essential oils they are called balsams) or oils are located in excretion or resin channels of the bark or wood of stems or roots.

The volatile fraction plays a fundamental role in regulating the interactions of a plant with its surrounding environment. Frequently, these fractions are known to have antimicrobial activity that could act as a chemical defence against plant pathogenic diseases. Terpenes may possess growth-regulating properties (e.g., abscisin) and are agents of protection and defense against phytopathogens and interactions between plants and animals (e.g., pheromones). The mono- and sesquiterpenes often occur as glycosides, too, and as such they are involved in essential oil metabolism and possibly serve as a transport system for the volatile compounds.

Sesquiterpene lactones are an important class of active compounds that possess a number of striking properties, such as a bitter or pungent taste and the ability to act as allergens, irritants, or poisons.

23.3 HPLC OF VOLATILE MONO- AND SESQUITERPENES

Although HPLC (high performance liquid chromatography) is not recommended for terpene analysis, attempts to monitor volatile terpenes are not lacking. The separation of enantiomeric volatile terpenes is difficult since they lack functional groups that provide the hydrogen-bonding, dipole, π–π, or charge-transfer interactions required for most chiral recognition mechanisms.

Cyclodextrins (CD) have been very successful in the separation of these compounds because of their ability to provide enantiomeric selectivity through an inclusion mechanism. The most successful technique used in the separation of volatile terpenes is their direct resolution by gas chromatography (GC) using CD bonded stationary phases [2,3]. However, the direct resolution of enantiomeric terpenes has already been achieved using HPLC with an α-CD bonded stationary phase [4] or using α-CD as a mobile-phase additive [5,6]. Several techniques have been used in the determination of apparent formation constants and stoichiometric coefficients of the guest-CD complex, such as nuclear magnetic resonance (NMR) spectrometry, potentiometry spectroscopic techniques, and reversed-phase HPLC (RP-HPLC) [7].

HPLC has proved to be a powerful technique for the investigation of the CD inclusion phenomenon. Moeder *et al.* [8] reported the determination of apparent formation constants and stoichiometric coefficients of terpene-CD inclusion complexes using HPLC with α- and β-CD as chiral mobile-phase additives. Four monoterpenes were studied, three of which (α-pinene, β-pinene, and camphene) are bicyclic in structure and one of which is monocyclic (limonene). Their stoichiometric coefficients of complexation and their apparent formation constants were determined. Chiral recognition, a result of the difference between the apparent formation constants of the enantiomers, was achieved for the bicyclic terpenes with α-CD. A difference in apparent formation constants was observed only when a 1:2 terpene-CD complex was formed. α-Pinene, β-pinene, and camphene formed a 1:2 terpene-α-CD complex; their enantiomers were resolved. Enantiomeric separation was not achieved using β-CD. The monocyclic terpene, limonene, was observed to form a 1:1 guest-α-CD complex and, likewise, its enantiomers were not resolved. All of the terpenes studied lacking functional groups that can interact with the secondary hydroxyl groups on the rim of the CD cavity. Therefore, instances of enantiomeric resolution can be attributed to steric differences between the enantiomers upon inclusion in the CD cavity. When these steric differences are pronounced, the observed apparent formation constant for each enantiomer-CD complex is different, and enantiomeric resolution is observed (Table 23.1).

23.4 APPLICATION OF HPLC TO QUALITY CONTROL OF FOOD ADDITIVES AND SPICES

23.4.1 Limonene–Perillic Acid

Volatile mono- and sesqiterpenes are found in most common fruits, especially in *Citrus* species. They are responsible for the flavor. *Citrus*-based terpenes (e.g., D-limonene) and oils can be used in a wide variety of applications, for example, as fragrances, air fresheners, cleaners, and fruit juices. Much analytical work has been done with regard to the differentiation of naturally grown aromatic compounds and those produced by commercial methods such as distillation or cold pressure.

The use of HPLC in the analysis and, particularly, in the isolation of flavoring constituents was introduced in the early 1980s. These methods have been reviewed by Bitteur [9]. Chamblee *et al.* have reported a general HPLC method for the prefractionation of the oxygenated terpenoic constituents of essential oils [10]. This method is not useful for terpene hydrocarbons, however, as they tend to coelute early without proper resolution. Several methods for separating terpene hydrocarbons by RP-HPLC have been reported [11,12], but the use of aqueous solvents in these methods requires time-consuming extraction before the samples can be concentrated and analyzed by GC–mass spectrometry (MS). Kubeczka [13] reported a normal-phase separation of mono- and sesquiterpene hydrocarbons at –15°C with pentane, which appeared cumbersome to practice.

TABLE 23.1
HPLC Conditions of Some Volatile Compounds and Saffron

Ref.	Column	Mobile Phase/Temp.	Detection	A: Derivatization B: Precolumn	Sample
[8]	Vydac C_4, 10 µm, 300 Å, 150 × 46 mm	0.1% H_3PO_4–methanol (55:45) + α- or β-CD	UV, 210 nm	—	α-Pinene, β-pinene, camphene, limonene
[14]	Partisil PXS, 5/10 µm, 250 × 4.6 mm; Radical-PAK, 5, 10 µm, 100 × 8 mm	Hexane	RI	B: Whatman HC-Pellosil, 70 × 2.1 mm	Germacrene, lime oil
[15]	Cosmosil 5 C_{18}, 150 × 4.6 mm	70% methanol, 37°C	UV, 517 nm	A: DPPH	Citrus oil, geraniol, terpinolene, γ-terpinene
[17]	Supelco LC-ABZ, 150 × 4.6 mm	Plasma: AN–25 mM NaAc buffer (pH 5.2); ratio 29:71 Lemonades: Ratio 70:30	UV, 230 nm	—	D-Limonene, perillic acid
[18]	LiChroCART 125-4 Supersper 100 RP18, 4 µm	Gradient program: AN–methanol (20–100%)	DAD, 308 nm	—	Picrocrocin, safranal
[19]	Millipore Novapack RP18, 250 × 4 mm	Gradient program: methanol–water (20–70%), 30°C	DAD, 310 nm	—	Picrocrocin, safranal
[20]	Nova-PaK C_{18}, 150 × 3.9 mm	Linear gradient: methanol–1% acetic acid (20–80%)	DAD, 250 nm	B: Econoshere C_{18}, 5 µm	Picrocrocin
[21]	Nucleosil C_{18}, 5 µm 125 × 4 mm	Gradient program: methanol–water (20–80%), 30°C	UV, 310 nm	—	Picrocrocin, safranal

Note: AN, acetonitrile; DAD, diode array detection; DPPH, 1,1-diphenyl-2-picrylhydrazyl; NaAc, sodium acetate.

There are two types of commercial lime oils (*Citrus aurantifolia* Swingle), expressed and distilled, on the market. The most widely used type is distilled lime, produced by crushing the whole fruit and steam distilling the oil. The other type is isolated directly from the peel of the fruit and is referred to as expressed, centrifuged, or cold pressed. These two types differ greatly from each other in their chemical composition and aromatic character. Clark *et al.* [14] reported a semipreparative, normal-phase HPLC method using three silica columns in tandem and elution with hexane at room temperature. It allowed both a very good separation of the higher hydrocarbon fraction of the lime peel oil and the isolation of a new lime constituent, germacrene B (Figure 23.3), which has been found to be an important compound for lime's flavor impact. Germacrene B has a potent, warm, sweet, woody-spicy, geranium-like odor and is very important to the fresh lime peel character of the oil.

The way the authors identified the germacrene B was very clever: Lime oil was distilled to yield a pot residue that contained non-GC-volatile material and appreciable amounts of the sesquiterpene hydrocarbons and sesquiterpene alcohols. This pot residue was separated by open-column LC on silica gel into a hydrocarbon and an oxygenated fraction. Evaluation of the hydrocarbon fraction by smelling the GC effluent led to the isolation and identification of germacrene B.

Antioxidants have been widely used in the food industry to extend the shelf life of foods that are susceptible to lipid oxidation. However, there are some arguments about the safety and adverse effects of synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene

(BHT), as food additives. That is the reason why—in recent years—many researchers have focused on natural antioxidants such as ascorbic acid and *R*-tocopherol. Today, natural antioxidants found in dietary plants play an important role in the prevention of carcinogenesis, cardiovascular problems, and various chronic diseases. These diseases are considered to be free radical-induced. Therefore, one important strategy in the prevention of these diseases is to neutralize or scavenge free radicals. There is increasing interest in the radical-scavenging activities of some natural antioxidants, especially those found in edible and many aromatic plants, especially in *Citrus* fruits. The latter and their essential oils have been known to support various biological activities such as antimicrobial properties and antioxidant, that is, radical-scavenging, capacity.

Choi *et al.* [15] studied the radical-scavenging activities of *Citrus* essential oils. Determinations of the radical-scavenging activities of *Citrus* essential oils and their components against the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) [16] were carried out by HPLC and compared with the activity of a standard antioxidant, Trolox. Trolox is a hydrophilic carboxylic acid derivative of *R*-tocopherol. DPPH has a dark violet color and a maximum absorption at 517 nm. The peak of the DPPH radical is decreased in the presence of a hydrogen donor, that is, a free radical-scavenging antioxidant. Thus, the free radical-scavenging effects of *Citrus* essential oils were investigated by evaluating the *decrease* in the peak height of the DPPH radical at 517 nm. It was found that *Citrus* volatile components such as geraniol (87.7%, 235.9 mg of Trolox equiv/mL), terpinolene (87.4%, 235.2 mg of Trolox equiv/mL), and γ-terpinene (84.7%, 227.9 mg of Trolox equiv/mL) showed marked scavenging activities on DPPH ($p < 0.05$).

Another very interesting component is D-limonene (Figure 23.4), a monoterpene that is widely distributed as a natural nonnutritive constituent of a variety of foods and volatile oils, particularly *Citrus* oils. D-Limonene and its derived metabolites have been shown to possess chemotherapeutic and chemopreventive efficacy against cancer in various preclinical model systems. The principal sources of D-limonene in the diet are the oils of orange, grapefruit, and lemon. D-Limonene (in the form of orange oil/essence oil) is also used as a flavoring ingredient for citrus flavor in artificial oils and can be found in beverages, ice cream, candy, and baked goods. In addition, both enantiomers, D- and L-limonene (the trivial name is dipentene), are used as a detergent.

The objective of the study presented by Chow *et al.* [17] was to determine whether D-limonene or its derived materials would be available systemically after the oral consumption of orange juice or lemonade. Because perillic acid (Figure 23.4) has been identified to be one of the major metabolites of D-limonene in humans and has been shown to exert potent biological activities, the study was designed to determine the systemic availability of perillic acid after a single-dose administration of orange juice.

The analysis of the D-limonene content of the juices or lemonades was performed using RP-HPLC procedures with a mobile phase that consisted of acetonitrile and sodium acetate buffer (pH 5.2) in the ratio of 29:71. The lemonade preparations were mixed and diluted with the mobile phase before injection onto the HPLC. Plasma perillic acid was detected at a wavelength of 230 nm, and the

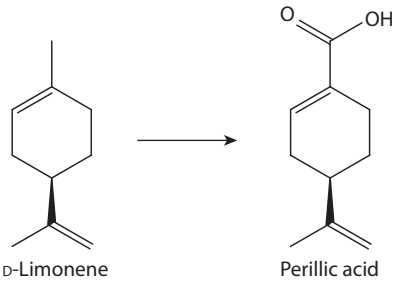


FIGURE 23.4 D-Limonene and its metabolite perillic acid.

amounts were quantified using calibration curves prepared with plasma, spiked with perillic acid standards (Table 23.1).

23.4.2 Saffron

The term *saffron* ("filaments") refers to the dried dark red stigma of *Crocus sativus* L. flowers. *Crocus sativus* plants (Iridaceae family) are principally grown in Spain and Iran but are nowadays cultivated in many other countries as well. Saffron is used both as a spice for flavoring and coloring food preparations and as a drug in medicine. It has also been used as a perfume ingredient. One stigma of saffron weighs about 2 mg, and each flower has three stigmata; 150,000 flowers yield 1 kg of spice. Due to its high value, saffron is susceptible to adulteration, usually by adding other plants (*Cuscuta planiflora* Ten., ligules of *Calendula officinalis* L., stigmata of *Zea mays* L.) or by adding other parts of the saffron plant itself (including styles, stamens, and strips of the corolla), which may be dyed with brazilwood, sandalwood, and artificial colorants (e.g., tartrazine).

The chemical composition of saffron has been thoroughly studied during the last decades by several groups. Among the estimated more than 150 volatile and several nonvolatile compounds of saffron, approximately 40–50 constituents have already been identified. The three main components in saffron are the colored water-soluble carotenoids named crocins, picrocrocin (a colorless and bitter-tasting sesquiterpene glycoside), and its water-insoluble aglycon, safranal (Figure 23.5). Safranal is the compound most responsible for the aroma of saffron spice and is, together with the suite of crocin pigments, the major determinant of the product quality. Picrocrocin and safranal are very sensitive compounds and may be degraded during the processing and storage of saffron, whereby the water-soluble picrocrocin is converted to the volatile and largely water-insoluble safranal either by a two-step enzymatic/dehydration process involving the intermediate HCC (4*R*-hydroxy-β-cyclocitral) or directly by dehydration at high temperatures > 60°C or extreme pH (6–10). The reported problems of measuring both the pigments and safranal in one single run at different wavelengths according to their differing UV maxima could be solved by introducing photodiode array detectors (DAD) to the analysis.

Extracts obtained by different extraction methods (maceration with 50% methanol in the dark, Soxhlet extraction with diethyl ether) were compared by Tarantilis *et al.* using RP18-HPLC separation and UV detection at different wavelengths: 280, 308, and 480 nm [18]. Some years later, an RP-HPLC method with DAD was developed by Lozano *et al.* to check the identity and purity of commercial saffron [19]. By this method, 10 saffron metabolites responsible for the taste, flavor, and color were identified and quantified with high selectivity, precision, and accuracy. The method

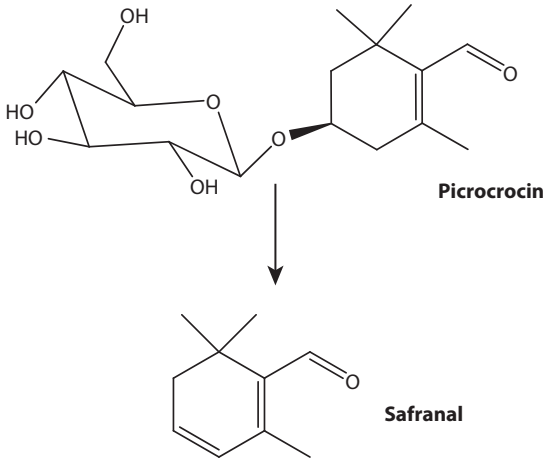


FIGURE 23.5 Picrocrocin and its aglycon safranal from saffron (*Crocus sativus* L.).

proved suitable to detect adulterants, such as artificial colorants (Tartracin, methyl orange, Ponceau S). The extraction of the saffron was performed using methanol–water (50%, v/v) with magnetic stirring during 1 h in the dark at 25°C. Before HPLC analysis, 4-nitroaniline (0.2 mg/mL) was added to the saffron extract as an internal standard. Three different saffron types were studied, and their metabolite concentrations determined at different wavelengths: picrocrocin and HCC at 250 nm, safranal at 310 nm, and all of the crocins and artificial colorants at 440 nm. A linear gradient elution of methanol (20–70%) at 1% methanol/min gradient speed and 1 mL/min flow rate was selected as the mobile phase.

Another paper [20] discussed the effect of drying temperature and air flow on the production and retention of secondary metabolites in saffron. Quantitative measurement using GC and HPLC-UV/Vis techniques was done to analyze the secondary metabolite contents of the products. The problems in measuring the concentration of both compounds—crocin and safranal—were solved by a dual-solvent extraction. For the determination of the pigments, picrocrocin, and possibly safranal, methanol was chosen; for comparison of safranal (and HCC) extraction, the nonpolar solvent hexane was used, and the quantification was attempted using GC analysis. The different results in HPLC and GC analysis are likely due to a combination of safranal's poor solubility/extraction in polar solvents and interfering absorbance at 330 nm by *cis*-crocins, tending to mask any differences that exist between different samples.

The aim of the investigations of Haghighi *et al.* [21] was to extend the capability of the previously reported HPLC method [19] by applying analysis of variance in chromatographic data to the detection of four types of adulterated saffron. The chromatograms were recoded simultaneously at three general wavelengths, 254, 330, and 440 nm, for the analysis of saffron metabolites and additional selected wavelengths, 402, 260, or 535 nm, for the analysis of saffron petals, safflower, madder, and red beet colorants, respectively (Table 23.1).

23.5 APPLICATION OF HPLC TO IDENTIFICATION AND QUALITY CONTROL OF PLANTS MONOGRAPHED IN PHARMACOPOEIAS

23.5.1 Arnica

The flower heads of *Arnica chamissonis* contain sesquiterpene lactones of the pseudoguaianolide type. Several helenanolides (Figure 23.6) were isolated and identified. Owing to their antiphlogistic and antibacterial properties, sesquiterpene lactones are the pharmacologically active compounds of the monographed Arnicae flos. The *European Pharmacopeia* (6th ed.—PhEur 6) [22] provides an HPLC method for the quantitative detemination of helenanolides using RP18 columns and a methanol–water gradient as the mobile phase and UV detection at 225 nm, whereby santonin is assigned for an internal standard.

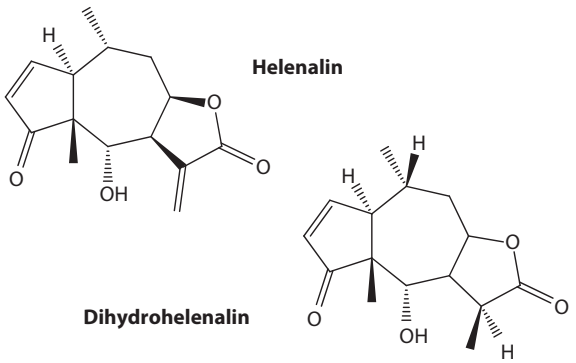


FIGURE 23.6 Helenanolides of *Arnica chamissonis.*

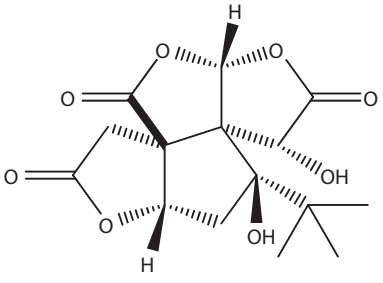
In earlier papers [23–25] this monograph has been prepared by thorough investigations. A method of sample preparation was presented to give the best results for extracting and concentrating the neutral, amphilic sesquiterpene lactones of *Arnica* flowers. The methanolic (50%) extract was concentrated and placed on an Extrelut column to separate the sample from interfering hydrophilic compounds. The HPLC analysis was performed using an RP column and a linear gradient of water and methanol as the mobile phase. This topic has been touched on again by Douglas *et al.* [26]. Moderate changes were carried out in terms of sample cleanup (SPE cartridges were used) and programming the mobile-phase gradient.

23.5.2 Ginkgo

Ginkgo biloba L. (Ginkgoaceae), a 40-m-high monotypic tree with typical fan-shaped leaves, has been valued for many centuries in Asia. Chinese traditional medicine uses the leaves (*bai guo ye*) and fruits (*baiguo*) to treat a number of diseases such as asthma, tuberculosis, and arteriosclerosis. In Western medicine, the leaves and special preparations thereof are utilized against dementia disorders, arterial occlusive disease, and vertigo. The ESCOP [27], and PhEur [22], and the U.S. Pharmacopeia and National Formulary (USP/NF) monographs [28] also list flavonoids and, above all, terpene trilactones (TTL) such as bilobalide (Figure 23.7) and ginkgolides as active ingredients. Ginkgolides, e.g., ginkgolide B, competitively inhibit the platelet-activating factor (PAF), thus preventing thrombus formation and bronchoconstriction and suppressing allergic reactions.

There are numerous commercial *Gingko* products: *Gingko* medicinal products are regulated in Europe under EU Regulation 2004/27 [29] and in the United States *Ginkgo* products are marketed as dietary supplements [30], all claiming to contain a specific amount of standardized extract per unit based on the content of flavonoids and terpene lactones. Due to the great difference in their concentration (0.5–1.8% for flavonoids and 0.03–0.25% of TTL in Gingko *leaves* versus >24% flavonoids and > 6% TTL in Gingko *extracts*, e.g., EGb 761, and products) and their chromatographic properties, these two classes of compounds are usually measured separately. The PhEur [22] demands only the HPLC determination of flavonoids, whereas the USP/NF [28] offers a method for measuring the content of TTL stipulating an RP column and a water–methanol gradient, followed by evaporative light scattering detection (ELSD).

However, the efficiency of extraction depends not only on the solubility of the substances but also on the desorption from the (plant) matrix; therefore, the majority of the earlier published methods focus on sample preparation and purification [31–35]. They differ in proposing the most effective extraction solvents, for example, water, methanol, acetone, or mixtures of them, even supercritical carbon dioxide. For sample purification the authors described the use of Extrelut or alumina cartridges. As an alternative to these conventional extraction techniques, a microdialysis system connected to the sample loop of the injector of the HPLC system was described [36]. Mauri *et al.* [37] applied an LC-APCI (atmospheric pressure chemical ionization)–MS system for measuring the



Bilobalide

FIGURE 23.7 The sesquiterpene bilobalide of *Ginkgo biloba* L.

amounts of TTLs in plasma of volunteers after administration of *Gingko biloba* extracts and getting pharmacokinetic data.

HPLC on RP columns soon became the method of choice for the analysis of *Gingko* extracts. Variations in eluent mixtures should overcome the difficulties in separating the TTLs from the coeluting components. The next problem was caused by the poor UV absorbance of the TTLs. To improve the detection limit, HPLC coupled with a UV detector was completed by a refractive index (RI) detector. Unfortunately, RI detectors are not suitable for gradient eluents. ELSD [38,39] and, particularly, MS detection have significantly enhanced the detection sensitivity for TTLs and the reliability of quantification [40,41]. Thus, qualitative and quantitative measurement is now possible without prior purification and enrichment of the compounds. These detection methods are valuable for research; nevertheless, the industry prefers RI or UV detection methods for routine analysis (Table 23.2).

23.5.3 Valeriana

Valerian root consists of the dried underground parts of *Valeriana officinalis* L. *s. l* (Valerianaceae). Valerian roots are monographed in PhEur 6, ESCOP, and USP/NF [22,27,28]. They contain not less than 5 mL/kg of essential oil calculated on the dried basis. The oil consists of monoterpenes (such as bornyl esters, camphene, and pinenes), sesquiterpenes (including valerenal and valeranone), and less volatile sesquiterpene carboxyclic acids such as valerenic acid and derivatives. Valerenic acid is a marker compound of *Valeriana officinalis* (Figure 23.8). The nonvolatile and instable valepotriates may be present in minor amounts; lignanes are likewise present and contribute, for example, along with valerenic acid to the soft calming and spasmolytic action. The content of valerenic acid has to be determined using a HPLC system consisting of an RP18 column, a mobile phase of a mixture of 80% methanol and 20% dilute (1 in 200) phosphoric acid, and a UV detector set at 225 nm.

Early attempts to optimize HPLC procedures in order to obtain a sensitive on-line HPLC method in which valerenic acid and its derivatives (as well as the valepotriates and the baldrinals) could be detected simultanously in one single run have been published. Bos *et al.* [42] performed an analysis of valerenic acid on an RP18 column using a gradient mixture of water and acetonitrile and DAD. The separated compounds could be detected by their UV spectra.

The goal of Komarova *et al.* was to isolate and identify valerenic acid as a standard substance from dry underground parts of *Valeriana officinalis* [43]. Isolation was executed by thin-layer chromatography (TLC), and HPLC analysis of valerian preparations was performed using an RP18 column with a methanol–phosphate buffer mixture, adjusted to pH 2, as the mobile phase and UV detection at 250 nm. Bicchi *et al.* embarked on a new way to analyze valerenic acid and valepotriates in extracts of *Valeriana officinalis* by p-SFC (packed column supercritical fluid chromatography)–UV and compared the results with those obtained by HPLC-UV [44]. The separation of these compounds by p-SFC was performed in the normal-phase mode using a CN modified column and supercritical carbon dioxide modified with methanol–water (95:5) as the eluent. The HPLC analysis operated in the reversed-phase mode using an RP18 column and a linear gradient mixture of water, acetonitrile, and phosphoric acid. The detection wavelength of the DAD was set at 225 nm for valerianic acid and 254 nm for valepotriates. The qualitative and quantitative results of p-SFC are comparable to those of HPLC for both valerenic acid and valepotriates, but analyses are faster with p-SFC than with HPLC (Table 23.3).

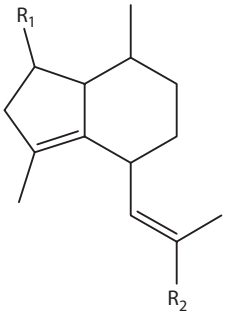
23.6 APPLICATION OF HPLC TO TRADITIONAL CHINESE MEDICINE (TCM): QUALITY CONTROL AND IMPROVEMENT OF CULTIVATION

Traditional Chinese medicine (TCM) has been practiced for many millennia, and over this long period of time, thousands of formulae have been verified based on their undoubted protecting and

TABLE 23.2
HPLC Conditions of *Arnica* and *Ginkgo* Samples

Ref.	Column	Mobile Phase/Temp.	Detec-tion	A: Cleanup B: Precolumn C: Sample Extraction	Sample
[22]	RP_{18}, 4 µm, 120 × 4 mm	Gradient program: 38–55–100% methanol in water	UV, 225 nm	—	Helenanolides, int. standard: santonin
[23]	Hypersil ODS, 5 µm, 125 × 4.6 mm or 250 × 4.6 mm	Gradient program: methanol–water I: 45–0% II: 50–55%	UV, 225 nm	A: Extrelut cartridge	Helenalin and other STLs of *Arnica*
[26]	LiChroCart 100 RP_{18}, 5 µm, 250 × 4 mm	Gradient program: 45–85% methanol in water	UV, 225 nm	A: SPE cartridge B: LiChrocart 100 RP_{18}, 5 µm, 4 × 4 mm	Helenalin and other STLs of *Arnica*
[28]	RP_{18}, 250 × 4.6 mm	Gradient program: 25–75–90% methanol in water	ELSD	—	Bilobalide, ginkgolides
[35]	Curosil PFP (pentafluoro-phenyl), 150 × 4.6 mm	Gradient program: 10–30–70–85% acetonitrile in water	ESI-MS	C: Supercritical fluid extraction	Bilobalide
[37]	Hypersil C_{18}, 5 µm, 100 × 3 mm	Gradient: 30–45% methanol in water	APCI-MS	—	Bilobalide in human plasma
[39]	Phenomenex Synergi Max RP, 4 µm, 150 × 4.6 mm	Gradient: methanol–isobutanol (9:1)–10 mM NH_4–acetate buffer (pH 5) (10–20–25%)	DAD, 205 nm; ELSD; MS	—	Bilobalide, ginkgolides
[40]	Microsorb Spherical ODS2, 3 µm, 100 × 4.6 mm	Methanol–water (29.5:70.5, v/v)	RI	B: ODS2 5 µm	Bilobalide, ginkgolides
[41]	Microsorb Spherical ODS2, 3 µm, 100 × 4.6 mm	Gradient: 20–40% methanol in water	RI, SSI-MS	B: ODS2 5 µm	Bilobalide, ginkgolides

Note: APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; ELSD, evaporative light scattering detection; MS, mass spectrometry; RI, refractive index detector; SSI, somic spray ionization; STL, sesquiterpene lactone;



R_1	R_2	
–H	–COOH	Valerenic acid
–OH	–COOH	Hydroxyvalerenic acid
–OAc	–COOH	Acetoxyvalerenic acid

FIGURE 23.8 Valerenic acid and derivatives of *Valeriana officinalis* L *s.l.*

TABLE 23.3
HPLC Conditions of *Valeriana* Drugs

Ref.	Column	Mobile Phase	Detection	Sample
[28]	RP18	Methanol H_3PO_4 dil. 1:200 (80:20, v/v)	UV, 225 nm	Valerenic acid
[42]	Superspher100 RP18, 250 × 4 mm	Gradient program: 45–100% B A: AN 156.4 + 800 g H_2O B: AN 625.6 + 200 g H_2O	DAD, UV spectra	Valerenic acid
[43]	Nucleosil C_{18} 250 × 4 mm	Methanol–phospate buffer (pH2)	UV, 250 nm	Valerenic acid
[44]	Hypersil C_{18} ODS, 5 µm, 200 × 4.6 mm	Gradient: 100 A → 100% B A: H_2O–AN–H_3PO_4 (80:20:0.05) B: same (20:80:0.05)	DAD, UV	Valerenic acid

Note: AN, acetonitrile; DAD, diode array detection.

therapeutic effects and have thus been widely accepted by Asian communities. Due to the increasing interest in the application of TCM products around the world, quality control has become an important issue. Traditional Chinese formulations often comprise multiple herbs, each of which contains many compounds that may be relevant to the putative activity. For example, in *Ginkgo* products (see Section 23.5.2), which are a popular dietary supplement in the United States and a licensed and regulated medicinal product in Europe, terpene trilactones (ginkgolides A, B, C, and J and bilobalide) and many other types of constituents such as biflavones have all been shown to possess important pharmacological activities and should be considered during quality assessment. Therefore, many attempts are required to evaluate the quality of TCM preparations.

23.6.1 Artemisia

Malaria continues to be a major health problem in many areas of the world. The control of malaria is becoming more difficult due to the increased resistance of *Plasmodium* strains to commonly used drugs such as chloroquine and mefloquine. Hence, there is an interest in traditional medicinal plants that have been used to cure fever and malaria. Artemisinin "qinghaosu" (Figure 23.9), isolated from the Chinese medicinal plant *Artemisia annua* L., is a novel antimalarial drug with a sesquiterpene lactone structure containing an internal endoperoxide linkage that is essential for the activity.

Total synthesis of artemisinin and its derivatives such as artemether, arteether, and artesunate (Figure 23.9) on a commercial scale is actually possible but not very efficient. These semisynthetic substances have been derived from the parent compound artemisinin, hence the growing interest in plants with high yields of artemisinin and in powerful techniques for monitoring the active substances. To achieve this goal, different strategies have been brought into focus. Another goal was finding the best (meaning simple, rapid, reliable, and cheap) method for analyzing the components of *Artemisia annua* and the active ingredients of the corresponding medicinal products. Finally, a sensitive analytical method for the determination of artemisinin and its derivatives in biological fluids, for example, human serum, is required.

Wallaart *et al.* [45] worked to find the chemotype with the highest level of artemisinin or to enhance the yield of artemisinin by inducing tetraploidy in *Artemisia annua* plants. The sesquiterpene levels, including artemisinin, increased but with a changed ratio of the individual sesquiterpenes. Although colchicine-induced tetraploidy did not lead to the initial goal of directly producing

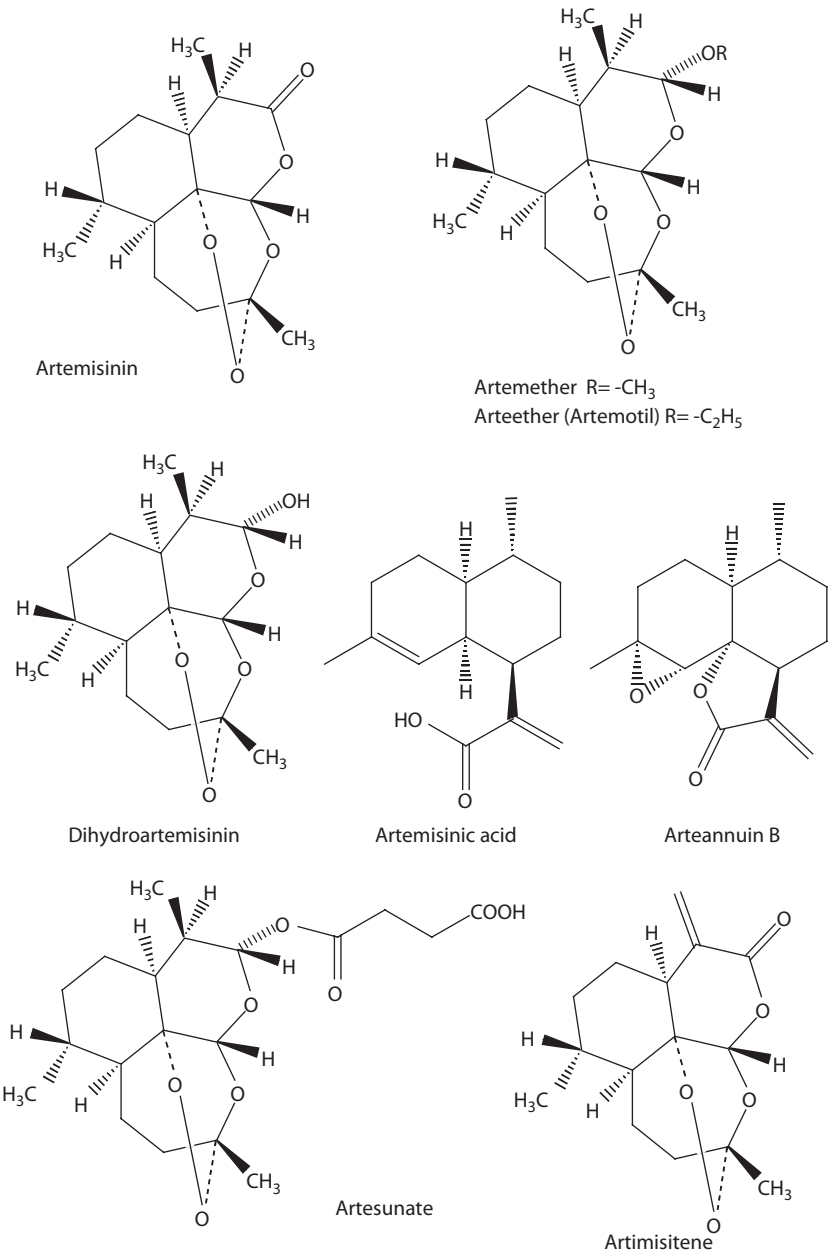


FIGURE 23.9 Artemisinin and its derivatives.

larger *A. annua* plants with higher artemisinin levels, tetraploid plants still could be used as a genetic bridge specimen for future breeding experiments to finally obtain an *A. annua* variety with an increased artemisinin production. The HPLC analysis was performed using an RP18 column and a mobile phase consisting of water–0.1 M H_3PO_4–acetonitrile in different ratios. The detection wavelength was set at 210 nm.

Wang *et al.* [46] examined the possible linkage between flowering time and the course of artemisinin biosynthesis. In fact, there was no direct linkage. To determine this, they performed the detection of artemisinin using an HPLC technique on an RP18 column with a mobile phase consisting of a mixture of sodium phospahte buffer and methanol, adjusted to pH 7.

Artemisinin (Figure 23.9) is UV transparent, but the endoperoxide bridge can undergo electrochemical (EC) reduction. Based on this property, an HPLC procedure with EC detection has been developed [47], using solid-phase extraction to clean up the plant extract. Artemisitene, a plant compound closely related to artemisinin, can be determined using UV detection, but the more sensitive EC detection, based on the endoperoxide, is preferred. Because molecular oxygen is also reduced, special precautions were required (using a modified injector) to deoxygenate the HPLC system, including the injected sample. A sample injected without degassing gave rise to a baseline destabilization of more than 50 min, probably due to oxygen saturation at the EC detector. The other related compounds (Figure 23.9) were detected by UV absorption at 228 nm since they lack an endoperoxide functionality. The EC and UV detections were performed simultaneously. All compounds were clearly resolved. The identification of the peaks in the leaf extract was performed by comparison to known reference standards using solvent systems with different ratios of sodium acetate (NaOAc) buffer and acetonitrile.

Peng *et al.* [48] presented the simultaneous analysis of artemisinin from plant samples by GC–flame ionization detection (FID) and HPLC-ELSD without sample derivatization. Here only the HPLC method will be discussed. The HPLC analysis was performed using a C_{18}-RP column, a mobile phase consisting of a mixture of acetonitrile and water, adjusted to pH 3.0–3.5 with trifluoroacetic acid, and ELSD. When the plant samples were analyzed, artemisinin eluted at 7.63 min, but in some samples of *Artemisia annua* plants no artemisinic acid or arteannuin B could be detected. This could be due to the high temperatures of the evaporator (80°C) and nebulizer (75°C) used for this HPLC-ELSD method. Decreasing the working temperatures (<40°C) led to the detection of artemisinic acid. Very low levels of artemisinic acid and dihydroartemisinic acid are better quantified by UV detection at 210 nm.

Artemisinin-derivative drugs are widely used to treat *Plasmodium falciparum* malaria, but very few studies have been performed to investigate the quality of these medicines in Africa. Atemnkeng *et al.* [49] analyzed the active ingredients of artemisinin-derivative drugs marketed in Kenya and Congo, containing either artemether, arteether, artesunate, or dihydroartemisinin (Figure 23.9). The content of active ingredients and preservatives was determined quantitatively using validated HPLC–UV methods. All analyses were done according to PhEur requirements.

Counterfeit or substandard artemisinin-derivative drugs are being sold in parts of Africa, presenting a potential route for resistance development in the future. Artemisinin derivatives are now available in the big cities of Africa and can be easily acquired without medical prescription. Unfortunately, fake products appear in all types of dosage forms. Therefore, quality-evaluation studies are primarily important to provide information on the drug content and to identify the cause (if any) of the poor quality. Thus, simple, rapid, and inexpensive assays are necessary to enable an easy setup of on-site quality control units. In Atemnkeng *et al.*'s study a total of 24 drugs were analyzed [49]. Only 15 (62.5%) met the PhEur content requirements of 95–105% of active drug substance. The analysis was performed by HPLC using reversed-phase C_{18} columns and mobile phases composed of acetonitrile–water–0.05 m KH_2PO_4. The detection wavelength was set at 215 nm.

Due to the remarkable properties of artemisinin and its derivatives, there was likewise increasing interest in the development of a simple, selective, and sensitive analytical method for the determination of artemisinin and its derivatives in biological fluids, such as human serum, to guide their right selection and rational use in different epidemiological situations. The method of Amponsaa-Karikari [50] is based on the fact that endoperoxide in artemisinin structure can be converted to hydrogen peroxide (H_2O_2) under UV irradiation, and the generated hydrogen peroxide can be measured using peroxyoxalate chemiluminescence (PO-CL) detection [51,52].

The HPLC-EC detection technique is also widely used for the determination of artemisinin in biological fluids because of its selectivity, sensitivity, and ability to detect both the parent drug (artemisinin, artemether, or arteether) and the major metabolite, dihydroartemisinin, simultaneously. The major drawback of this technique is the experimental rigor required to maintain a low level of oxygen in the HPLC system and the sample solutions, which is essential for the

TABLE 23.4
HPLC Conditions of *Artemisia* Drugs

Ref.	Column	Mobile Phase/Temp.	Detection	A: Postcolumn, B: Sample Extraction	Sample
[45]	Lichrosorb 7 RP18, 100 × 3 mm	H_2O–0.1 M H_3PO_4–AN (49:1:50, v/v)	DAD UV, 210 nm	B: Alkaline hydrolization	Artemisinin
[47]	Zorbax SB-CN, 250 × 4.6 mm	AN–0.1 M NaOAc (pH 5) (40:60, v/v)	UV 228 nm, EC	—	Artemisinin
[48]	Purosphere C_{18}, 5 µm	H_2O (pH 3–3.5)–AN (65:35, v/v)	ELSD, 80°C; 75°C	—	Artemisinin
[49]	Nucleosil 120 C_{18}, 5 µm, 125 × 4 mm;	AN–H_2O– KH_2PO_4 (0.05 M, pH 5.0) buffer (480:100:320, v/v/v)	UV, 215 nm	—	Artemisinin
[49]	LichroCart 100 RP18, 5 µm, 250 × 4 mm	AN–0.05 M KH_2PO_4 (pH 5.0) buffer (600:500, v/v).	UV, 215 nm	—	Artesunate
[50]	Supelco Discovery HS C_{18}, 250 × 4.6 mm	Imidazole–HNO_3 buffer (20 mmol/L, pH 8.50) containing 70% AN	UV, 254 nm	A: PO-CL: 0.50 mmol/L DNPO and 1.50 µmol/L TMP in AN; UV photoreaction 45°C	Artemisinin

Note: AN, acetonitrile; DNPO, (2,4-dinitrophenyl) oxalate; ELSD, evaporative light scattering detection; NaOAc, sodium acetate; PO-CL, peroxyoxalate chemiluminescence; TMP, 2,4,6,8-tetrathiomorphorinopyrimido [5,4-*d*] pyrimidine.

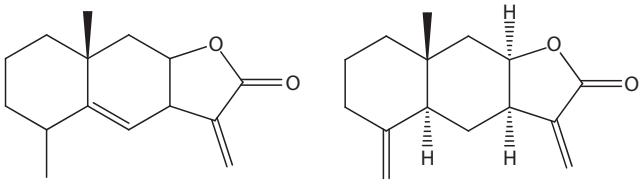
sensitive and stable operation of the EC detector in the reductive mode [53]. In the proposed method, artemisinin was eluted from the HPLC column and UV irradiated to generate H_2O_2. The generated H_2O_2 reacted with bis (2,4-dinitrophenyl) oxalate (DNPO) to form a high-energy intermediate that transfers its energy to 2,4,6,8-tetrathiomorphorino-pyrimido [5,4-*d*] pyrimidine (TMP), a coexisting fluorophore, and leaves TMP in an excited state. The excited TMP returns to the ground state by emitting light. The total light emitted is proportional to the concentration of artemisinin (Table 23.4).

23.6.2 Inula

The genus *Inula* (Asteraceae; tribe Inuleae) comprises several species of reputed medicinal value (*Inula helenium* L., *I. racemosa* Hooker fil., *I. viscosa* (L.) Aiton, and *I. britannica* L). About 100 species of the genus *Inula* are found widely in Europe, Asia, and Africa. Many of these have long been used in Chinese folk medicine, most frequently for their peptic, antibronchitis (phlegm relief), anti-inflammatory, and vermifuge properties. Sesquiterpene lactones (refer to Section 23.7.1) are the main secondary metabolites in this genus.

"Elecampane" (*Inula helenium* L.) is a widespread medicinal plant native to Middle Asia. Roots of the plant contain an essential oil with eudesmane-type sesquiterpene lactones, mainly the isomers alantolactone and isoalantolactone (Figure 23.10), thymol derivatives, triterpenes, sterols, and up to 44% of the polysaccharide inulin. The sesquiterpene lactones show anti-inflammatory activity and cytotoxic or antiproliferative activity against human cancer cell lines.

Roots of *Inula royleana* DC., another species native to Asia, have likewise been reported to contain alantolactone and isoalantolactone as major secondary metabolites. As sesquiterpene lactones



Alantolactone Isoalantolactone

FIGURE 23.10 The sesquiterpenes alantolactone and isoalantolactone from *Inula helenium* L.

TABLE 23.5
HPLC Conditions of *Inula* Samples

Ref.	Column	Mobile Phase/Temp.	Detection	Sample
[58]	Purosphere RP18, 5 μm, 125 × 3 mm	Methanol–water (3:2, v/v)	UV, 205 nm	Alantolactone, isoalantolactone
[59]	Alltech, Si Econosil, 10 μm, 250 × 7.8 mm	Hexane–$CHCl_3$–EtOAc (6:3:4, v/v/v)	UV	Ergolide, bigelovin

Note: EtOAc, ethyl acetate.

are, in general, thermolabile compounds of low volatility, HPLC is the appropriate analytical technique for their determination in plant extracts. The isomeric pair that accounts for >90% of the sesquiterpene lactone fraction in *I. royleana* and *I. helenium* roots [54,55] was not separable under standard RP-HPLC conditions. However, a number of techniques to separate the isomers have been described in the literature [56]. Hara *et al*. achieved a separation of these compounds using a packed microcapillary normal-phase HPLC column [57]. Stojakowska presented an isocratic RP-HPLC method that provides a simple and rapid quantification of the eudesmanolides and thymol derivatives in extracts from roots of *Inula* species using a mixture of methanol–water (3:2, v/v) as the mobile phase and UV detection at 205 nm [58]. Park and Kim presented a cytotoxicity-guided fractionation of the flowers of *Inula britannica* that led to the isolation of four sesquiterpene lactones, 4α,6α-dihydroxyeudesman-8β,12-olide, ergolide, 8-epi-helenalin, and bigelovin, with cytotoxic activity against human tumor cell lines [59]. The $CHCl_3$ extract showed the highest cytotoxicity (ED_{50} 1.9 mg/mL) in HL-60 cells. The extracts were fractionated and analyzed using normal-phase HPLC and different solvents (*n*-hexane, acetone, $CHCl_3$, isopropyl alcohol) in gradient and isocratic modes (Table 23.5).

23.7 APPLICATION OF HPLC TO DETERMINE PHARMACOLOGIC AND TOXIC PLANT INGREDIENTS

23.7.1 Sesquiterpene Lactones

23.7.1.1 Sesquiterpene Lactones with Toxic and Sensitizing Properties

Picrotoxin (PCX) is a compound in the plant *Anamirta cocculus* L. of the Menispermaceae family. Its convulsive effects were first described in 1875. Chemically, picrotoxin is an equimolar mixture of two sesquiterpene lactones, picrotin and picrotoxinin (Figure 23.11). Although PCX has been used in the treatment of barbiturate poisoning, at present, PCX is often used to block the activity of neuronal GABA and glycine receptors. However, the mechanism by which picrotoxin inhibits these receptors is still debated.

Soto-Otero *et al.* reported an RP-HPLC procedure for the quantification of picrotin and picrotoxinin in serum and, in addition, its successful application to evaluate the pharmacokinetics of

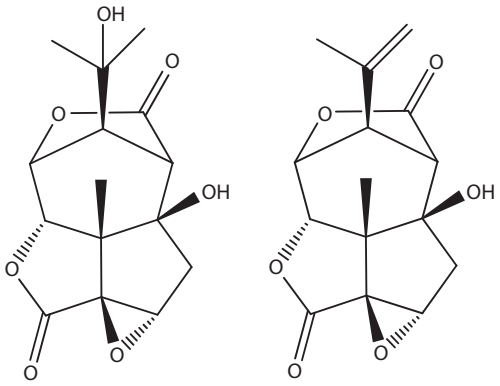


FIGURE 23.11 Picrotoxin of *Anamirta cocculus* (L.) as an equimolar mixture of the sesquiterpene lactones picrotin and picrotoxinin.

these two compounds after the administration of a convulsive dose of PCX to rats [60]. The chromatography was performed at room temperature with a mobile phase consisting of acetonitrile and 1 mM ammonium acetate buffer (pH 6.4) at a ratio of 34:66 (v/v); the detection wavelength was set at 200 nm.

Certain Asteraceae (syn. Compositae) families are harmful or toxic to cattle. Among them are such well-known livestock-poisoning plants as *Helenium*, *Hymenoxis*, *Inula*, and *Artemisia* species (refer to Section 23.6), which contain sesquiterpenes with an α-methylene γ-lactone structure (Figure 23.12).

A rapid and reproducible method for the quantitation of hymenoxone and related sesquiterpene lactones has been developed using RP-HPLC [61]. Hymenoxone and related sesquiterpene lactones were eluted within 10 min by RP-HPLC using either a C_{18} or RP-8 column. The quantitation of all the previously mentioned compounds was readily accomplished on the RP-8 column eluting with 50% methanol in water; however, the application of a C_{18} column was limited to the quantitation of helenalin and mexicanin E using 55–60% methanol in water.

HPLC analysis of *Helenium microcephalum* methanol extract readily separated helenalin and mexicanin E; however, when a methanol extract of *Helenium amarum* was analyzed, the separation of helenalin and tenulin was poor under the same conditions (R_t 7.13 and 6.59 min, respectively). The detection limits for the sesquiterpene lactones varied between 25 ng (for helenalin) and 75 ng (for hymenoxon). However, the detection limit could be lowered (approximately fourfold) by changing the monitoring wavelength to 220 nm.

Many species of the genus *Artemisia* (Asteraceae), for example, *Artemisia cina* Berg, *Artemisia caerulescens*, and *Artemisia maritima* L., are known for their anthelmintic properties, which are due to the presence of the sesquiterpene lactone α-santonin. Their use as an anthelmintic for humans has been displaced by safer products, though they are still used for pesticides and some veterinary products.

In 1998, Miraldi *et al.* [62] proposed a HPLC procedure for the determination of santonin, stipulating a LiChrospher 100-RP column and a mobile phase consisting of an acetonitrile–water gradient. The separation of santonin from other (unknown) compounds was satisfactory, and the UV detection and identification of santonin at 236 nm succeeded in comparing its retention time (R_t 14 min) to reference compounds.

Compositae dermatitis is an allergic dermatitis caused by plant species of the Asteraceae (syn. Compositae) family. Case reports of contact allergic ragweed dermatitis appeared in the U.S. literature as early as 1919. Ragweed and *Parthenium* dermatitis became prototypes for the classic, so-called airborne Compositae dermatitis. That affects primarly exposed skin surfaces and produces a universal erythroderma. Besides their toxic properties, sesquiterpenes containing an α-methylene

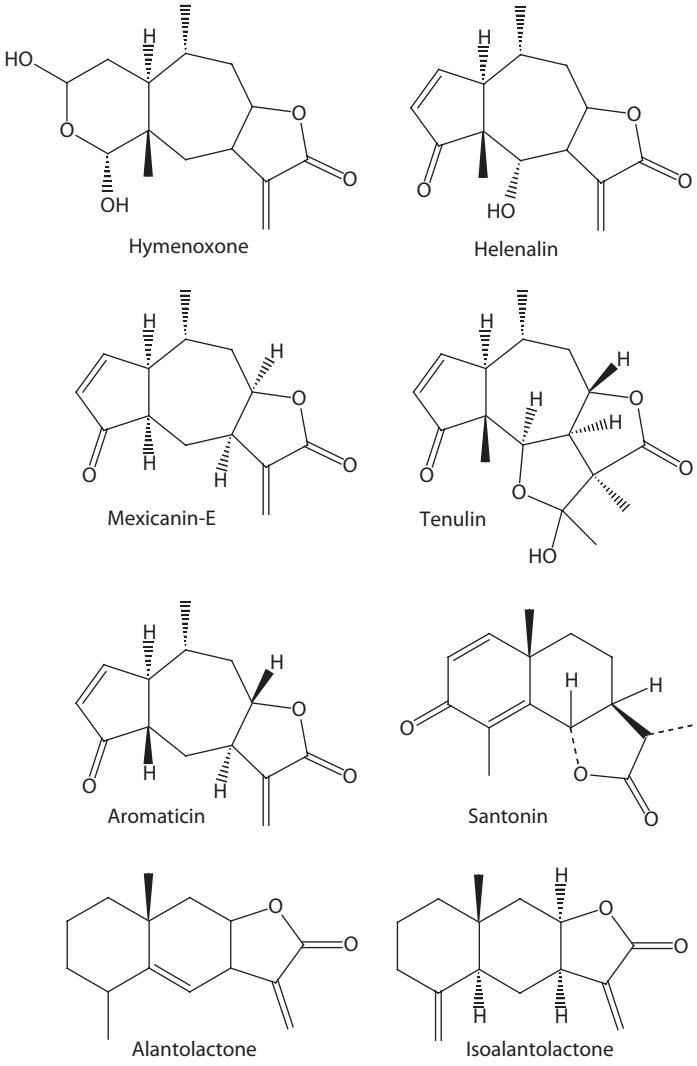


FIGURE 23.12 Sesquiterpene lactones with toxic properties. Samples 1–3 display the prominent α-methylene γ-lactone structure.

γ-lactone group (Figure 23.12) are the main sensitizers of the Asteraceae family. The photoreactivity of these sesquiterpene lactones is directed toward the DNA base thymine, which are producing intermolecular photoadducts operating as antigens within the cell. Photosensitivity is present in 22–75% of individuals sensitive to Compositae.

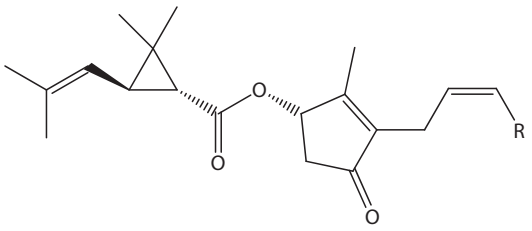
Among cultivated Asteraceae plants, *Tanacetum* (syn. *Chrysanthemum*) is considered to be a major sentitizer in Europe. Edible types are lettuce (*Lactuca sativa*) and endive (*Cichorium endivia*), and wild-growing plants are feverfew (*Tanacetum parthenium* syn. *Chrysanthemum parthenium*), tansy (*Tanacetum vulgare*), and dandelion (*Tanacetum officinale*). Tansy, pyrethrum, and most of the other species in the genus *Tanacetum* were previously classified as belonging to the genus *Chrysanthemum*, and a few authors still hesitate about their taxonomical status.

Pyrethrum, *Tanacetum cinerariifolium* (Trevir.) Schultz-Bip (syn. *Chrysanthemum cinerariifolium*), originates from Albania and the area of the former Yugoslavia. Formerly, the white *Chrysanthemum* flowers, pyrethri flos, could be found for sale in most European pharmacies as "Dalmatian insect powder." The primary uses for the pyrethrum flowers were for the control of

body lice on humans and animals and of crawling insects in the home. Pyrethrum produces insecticidal compounds collectively termed as pyrethrins, which are a combination of six esters: cinerin I, jasmolin I, pyrethrin I, cinerin II, jasmolin II, and pyrethrin II (Figure 23.13). Pyrethrins cause a rapid knock down and paralyzing effect on arthropod nerves. Sesquiterpene lactones in pyrethrum flowers have also inhibiting activity on seed germination.

Pyrethrin I is derived from an irregular monoterpene, chrysanthemic monocarboxylic acid, whereas pyrethrin II is derived from chrysanthemic dicarboxylic acid (pyrethric acid). The insecticidal activity increases with the increased ratio of pyrethrin I/pyrethrin II, which varies between 0.9 and 1.3 depending on the origin of the pyrethrins.

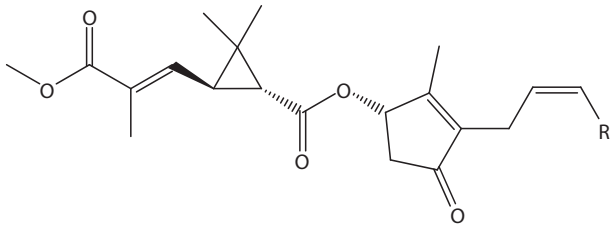
Earlier procedures for analysis of *Pyrethrum* extracts were based on titration, UV spectrophotometric detection, GC, TLC, and normal-phase HPLC methods. Due to the thermal instability of pyrethrins, TLC [63] and HPLC methods are preferred over GC methods for analyzing pyrethrins and related compounds. Initially, papers presented a normal-phase HPLC method of analysis using the solvent system *n*-hexane–tetrahydrofuran (96:4) with an increasing flow rate from 0.7 mL/min up to 2.3 mL/min [64]. Pyrethrins were detected by absorbance at 229 nm. Soon, RP-HPLC was introduced to the analysis of pyrethrins. Wang [65] proposed an RP-HPLC method for the direct determination of the six pyrethrins in pyrethrum extracts using a ternary mobile phase comprised of acetonitrile, methanol, and water as a gradient at a constant flow rate and DAD. This method was simplified by using a binary gradient elution program. The initial 50:50 volume ratio of the solvents acetonitrile and methanol was gradually changed to 65:35. The temperature of the column compartment was maintained at 40°C. Under these conditions the six pyrethrin esters were well resolved. To maximize detection and minimize any interferences, as far as possible, DAD was selectively performed at 230 nm (0–24 min), 240 nm (23–29 min), and 230 nm (>29 min). The sequence of separation was cinerin II (16.7 min), pyrethrin II (17.5 min), jasmolin II (19.9 min), cinerin I (26.3 min), pyrethrin I (27.5 min), and jasmolin I (32.2 min).



Pyrethrin I R = CH = CH_2

Cinerin I R = CH_3

Jasmolin I R = C_2H_5



Pyrethrin II R = CH = CH_2

Cinerin II R = CH_3

Jasmolin II R = C_2H_5

FIGURE 23.13 Pyrethrins with insecticidal properties from *Chrysanthemum cinerariaefolium*.

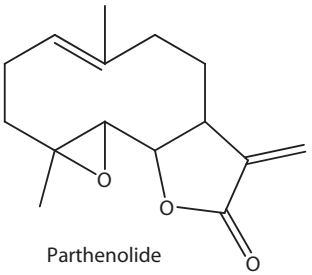


FIGURE 23.14 Parthenolide from *Tanacetum parthenium* (L.) SCHULTZ BIP.

Kasaj *et al*. [66] have developed and validated an RP-HPLC method for the quantification of total and individual amounts of pyrethrins from pyrethrum extract and flowers. The mobile-phase components acetonitrile (solvent A) and water (solvent B) were used for gradient elution. The detection wavelength was set at 230 nm. The individual pyrethrins were isolated from an extract by preparative normal-phase HPLC using hexane–ethyl acetate (93:7, v/v) as the mobile phase. The pyrethrins were detected at 255 nm.

Although most papers describe an excellent separation and resolution of the six pyrethrins using RP-HPLC methods and the current USP [28] stipulates an RP-HPLC analysis on a C_{18} ODS column with a methanol–water (80:20) mobile phase for pyrethrum extracts, some authors found fault with those results and decided at least to pursue a normal-phase HPLC methodology for the determination of the individual pyrethrins [67]. The mobile phase used was a mixture of hexane and tetrahydrofuran (97.75:2.25), and the detector wavelength was set at 240 nm. With the conditions outlined in their method, all six esters in the pyrethrum extract were baseline-separated. The t_R values were given as 7.371 min (for jasmolin I), 8.049 min (cinerin I), 9.243 min (pyrethrin I), 18.252 min (jasmolin II), 20.156 min (cinerin II), and 23.864 min (pyrethrin II).

23.7.1.2 Feverfew

Feverfew (*Tanacetum parthenium* L. Schultz-Bip.), a herbaceous member of the Asteraceae (Compositae), has a long history of use as a treatment for ailments, including skin disorders and urogenital complaints. Currently, the most extensive use of the leaf of the plant is for migraine prophylaxis. Studies with blood cells and platelets have implicated the germacrene sesquiterpene lactone parthenolide (Figure 23.14) as a pharmacologically active component. Clinical trials have confirmed that dried feverfew preparations with defined amounts of parthenolide have beneficial effects in migraine therapy.

Feverfew contains several sesquiterpene α-methylene γ-lactones and mainly parthenolide in the European species. In fact, parthenolide is the predominant sesquiterpene lactone found in British- and German-grown feverfew, but no parthenolide was found, for example, in Mexican-grown feverfew.

The effectiveness of various preparations of feverfew at inhibiting, in vitro, the release of serotonin from human blood platelets has been found to correlate well with levels of parthenolide. Therefore, numerous studies have reported on the quantification of parthenolide in feverfew leaves and preparations. Earlier procedures for determinations included infrared (IR) spectroscopy, TLC, and normal-phase HPLC. An RP-HPLC method for quantification using a mobile phase of water–acetonitrile (55:45) was presented in 1991 by Awang *et al.* [68]. The parthenolides were detected at 210 nm.

There have been no reports on the determination of parthenolide (*Tanacetum parthenium* Bernh.), marrubiin (*Marrubium vulgare* L.), and artemisinin (*Artemisia annua* L.) by normal-phase HPLC that could count as rapid and cheap. Therefore, Rey *et al.* presented a paper describing a rapid and inexpensive process for the isolation of the pure lactones and also described a normal-phase HPLC method for their determination in the aerial parts of the plants [69]. The mobile phase was

TABLE 23.6
HPLC Conditions of Sesquiterpene Lactones

Ref.	Column	Mobile Phase/Temp.	Detection	A: Cleanup B: Precolumn	Sample
[60]	Spherisorb ODS (C_{18}), 5 µm, 250 × 46 mm	AN–1 mM NH_4–acetate buffer (pH 6.4; 34:66, v/v)	UV, 200 nm	B: CoPell ODS 50 × 46 mm	Picrotoxin
[61]	RP8 or RP18	Methanol–water, 50% or 60% methanol, isocratic	UV, 220 nm and 235 nm	—	Hymenoxone, mexicanine E, helenalin
[62]	LiChrospher 100-RP, 240 × 4 mm	AN–water; gradient program: (4:6) to (6:4, v/v)	UV, 236 nm	B: LiChrocart 4-4	α-Santonin
[64]	Waters silica 10 µm Rad-Pak	Hexane–THF (96:4, v/v), isocratic	UV/Vis, 229 nm	B: Guard-Pak 10 µm	Pyrethrins
[65]	Restek C_8, 5 µm, 150 × 4.6 mm	Gradient: AN–water (1:1) up to (65:35, v/v), 40°C	DAD, 230 and 240 nm	—	Pyrethrins
[66]	Necleosil 100-5 C_{18}, 250 × 4 mm	A: AN B: water gradient program: from 58% A to 100% A	DAD, 230 nm	—	Pyrethrins
[67]	Spherex CN, 5 µm, 250 × 4.6 mm	Hexane–THF (97.75:2.25 or 98:2, v/v)	DAD 240 nm	—	Pyrethrins

Note: AN, acetonitrile; DAD, diode array detection; THF, tetrahydrofuran.

hexane–dioxane (85:15, v/v) at a flow rate of 1 mL min^{-1} for parthenolide and marrubiin elution, and hexane–dioxane (90:10, v/v) at a flow rate of 2 mL/min for artemisinin elution. UV detection was set at 210 nm for parthenolide and artemisinin and 225 nm for marrubiin.

Brown *et al*. stated that a crucial prerequisite for the accurate measurement of parthenolide and related compounds is an efficient extraction procedure involving either an initial organic or aqueous phase [70]. Their assay for parthenolide, involving an initial extraction of plant tissue with acetone followed by re-extraction of material in 100% ethanol, has a number of advantages. HPLC was performed using an RP18 column and a mobile phase of acetonitrile–water (40:60, v/v) at 2 mL min^{-1}. Peaks were analyzed at 210 nm.

Apparently, little had been done to find an efficient solvent system to quantitatively extract parthenolide in feverfew. The study of Zhou *et al*., which describes the extraction of parthenolide from feverfew, may provide this [71]. They found acetonitrile–water (90:10, v/v) combined with bottle stirring to be the best extraction system. The analysis was performed using an RP-HPLC system equipped with DAD. The mobile phase consisted of acetonitrile–water (55:45, v/v) at a flow rate of 1.5 mL/min, and parthenolide was detected at 210 nm with $R_t \approx 2.95$ min (Table 23.6).

23.7.2 Sesquiterpene Esters

Butter bur, *Petasites hybridus* (L.) G. M. et Sch. (Asteraceae), is a traditional herb used in European phytotherapy. Two chemotypes of the species, the furanopetasin and the petasin chemotype (Figure 23.15), are known. The two subtypes can be distinguished only by analytical means and not by morphological characteristics. The furanopetasin variety predominates in eastern Europe. In Switzerland the petasin variety seems more common. The petasin variety has been known since 1951 for its spasmolytic and analgesic activity. The major active compound was proved to be the eremophilane sesquiterpene ester petasin.

Debrunner [72] presented an HPLC method that allowed an efficient gradient separation of 14 genuine sesquiterpenes from rhizomes, roots, runners, buds, leaves, and stalks of *Petasites*

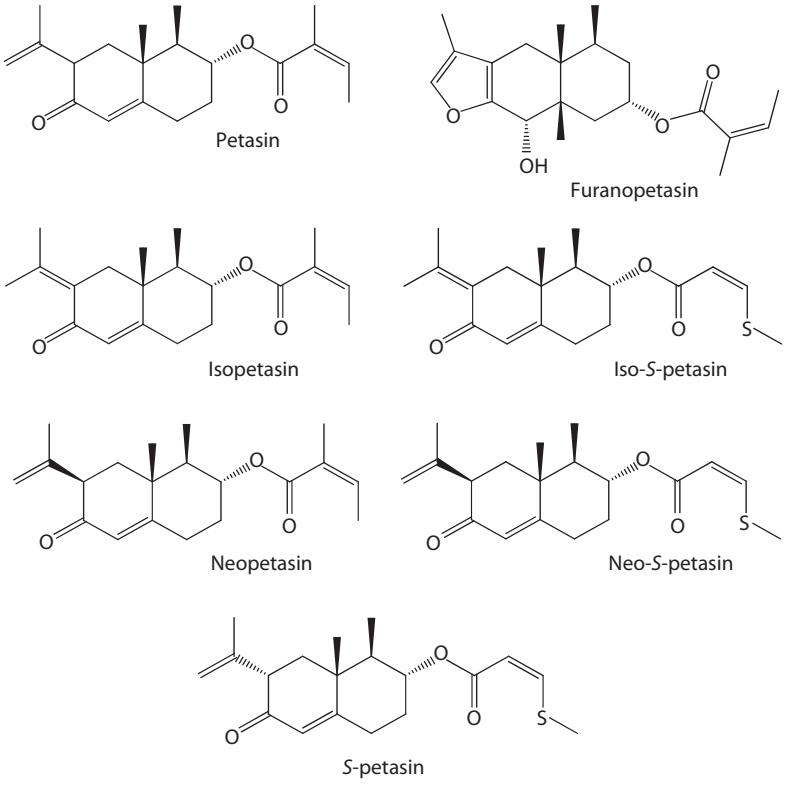


FIGURE 23.15 Petasin and isopetasin from *Petasites* species.

TABLE 23.7
HPLC Conditions of Sesquiterpene Esters of *Petasitis hybridus* (L.)

Ref.	Column	Mobile Phase/Temp.	Detection	A: Cleanup B: Precolumn	Sample
[72]	Nucleosil-100 3 µm, 250 × 4 mm	A: Hexane B: DIPE–AN (9:1, v/v), gradient	DAD, 254 nm	B: Nucleosil-100, 5 µm, 11 × 4 mm	Petasin and derivatives
[73]	Nucleosil 120-C_{18}, 3 µm, 250 × 4.6 mm	Methanol–AN–water (32:31:37, v/v/v), isocratic	DAD, 230 nm		Petasin

Note: AN; acetonitrile; DAD, diode array detection; DIPE, diisopropylether.

hybridus L. The sesquiterpene esters are separated on a normal-phase HPLC column with hexane–diisopropylether–acetonitrile gradients as the mobile phase and DAD at 254 nm. The six main compounds, isopetasin, neopetasin, petasin, iso-S-petasin, neo-S-petasin, and S-petasin, have been quantitatively determined. These compounds are abundant in all subterranean parts. Wildi *et al.* [73] screened about 14 populations of *Petasites hybridus* species for quantification of petasin using RP-HPLC. The methylene chloride extracts were separated on a C_{18} column using methanol–acetonitrile–water (32:31:7) as the mobile phase in an isocratic mode at a flow rate of 1 mL min^{-1} at 30°C. Petasin was detected at 230 nm by DAD. Analysis time was about 60 min (Table 23.7).

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24 HPLC Analysis of Diterpenes

Michał Ł. Hajnos

CONTENTS

24.1 INTRODUCTION

Diterpenes—C_{20} terpenes—are a big group of compounds that consist of 4 five-carbon (C_5) units called active isoprene. These compounds play an important role in the physiology of a plant and are also known for their pharmacological and toxicological activities. The group of diterpenes contains about 800 different substances with a known structure, occurring mainly in higher plants (sometimes in oleoresins and latices), microorganisms, and marine products. In phytochemistry, diterpenes are known as bitter substances, toxic diterpene alkaloids, plant growth hormones (gibberellins), other secondary metabolites, and, for instance, vitamin A (retinol), which shares the characteristics of the diterpene structure and occurs in human and animal organisms. Moreover, it has been discovered that pro-vitamin A—the group of tetraterpenoid compounds (carotenoids)—is present in plants. Diterpene compounds can reveal linear or mono-, di-, tri-, tetra-, and polycyclic (macrocyclic) and miscellaneous structures. From a chemotaxonomical point of view, some groups of diterpenes are common in the plant kingdom, while others are specific for small systematic groups such as families or genera.

24.2 CHEMICAL STRUCTURES OF DITERPENES AND DITERPENOIDS: BASIC CLASSIFICATION OF THIS GROUP OF COMPOUNDS

Diterpenoids constitute a vast class of isoprenoid natural products, biosynthesized from mevalonic acid through geranylgeranyl pyrophosphate (GGPP; see Figure 24.1). They can be classified as acyclic (phytane), monocyclic (retinol—vitamin A), bicyclic (labdane, clerodane), tricyclic (abietane, pimarane, rosane, podocarpane, chinane), tetracyclic (kaurane, trachylobane, beyerane, atisane, scopadulane, gibberellane), macrocyclic (cembrane, taxane, daphnane, tigliane, ingenane, jatrophane), and other miscellaneous compounds, also known as prenylsesquiterpenes (meroditerpenes, xenicanes, lobanes, prenylguaianes, dictalanes). Meroditerpenes, which are polycyclic diterpenes of mixed biogenesis characterized by a hydroquinonic methyl nucleus linked to a diterpenic chain, can serve as a good example here.

Ginkgolides and diterpene alkaloids are other groups of diterpenes. Since alkaloids are thoroughly discussed in the chapter on various groups of alkaloids, this type of diterpenes is only mentioned here.

The structures of the main diterpene groups are shown in Figures 24.2 through 24.10.

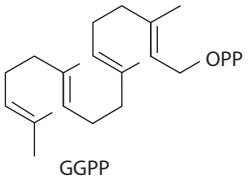

FIGURE 24.1 Geranylgeranyl pyrophosphate (GGPP) and octaprenyldiphosphate group (OPP).

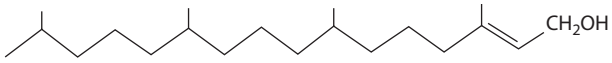

FIGURE 24.2 Phytane diterpene (acyclic) structure.

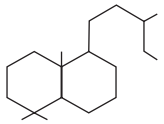

FIGURE 24.3 Labdane structure.

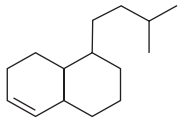

FIGURE 24.4 Clerodane structure.

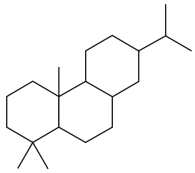

FIGURE 24.5 Abietane structure.

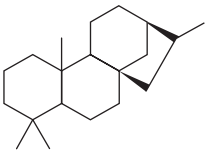

FIGURE 24.6 Kaurene structure.

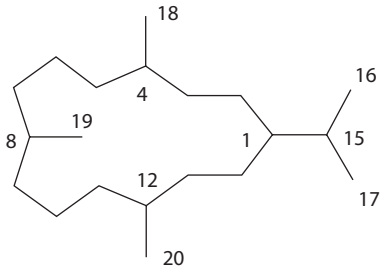

FIGURE 24.7 Cembrane structure.

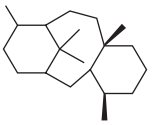

FIGURE 24.8 Taxane structure.

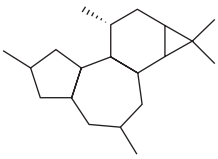

FIGURE 24.9 Tigliane structure.

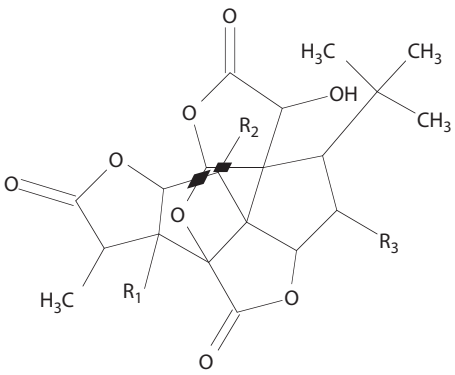

FIGURE 24.10 Ginkgolide structure.

24.3 NATURAL SOURCES OF DITERPENE COMPOUNDS

In some plant families, especially the Euphorbiaceae and Labiatae families, there are a lot of diterpenes with various skeletons. The Euphorbiaceae family contains the diterpenes of the ingenane, clerodane, tigliane, jatrophane, lathyrane, labdane, and daphnane groups, known for their irritant and toxic properties [1–3], whereas *neo*-clerodane, clerodane, labdane, and abietane diterpenes occur in the Labiatae family [4–6]. There are also plant families, such as the Asteraceae family, in which only several genera contain diterpenes. Diterpenes are also characteristic for resins occurring in the Pinaceae family (abietanes) [7]. For a change, taxoids and ginkgolides are unique compounds present only in the *Taxus* genus and *Ginkgo biloba* respectively. Diterpenes (*ent*-kaurenoids) are also found in ferns (Pteridaceae family) [8]. Completely different diterpenes, such as the briarane type and meroditerpenes, can be found in marine products (e.g., corals) [9,10]. Cembranolide diterpenes are also found in corals [11], whereas spongianes are present in sponges [12] and dolabellane and dolastane in mollusks [13].

24.3.1 Higher Plants

Division Marchantiophyta (liverworts)—Class Jungermanniopsida—Order Jungermanniales:

- Jungermanniaceae—*Jungermannia infusca* [14]

Division Pteridophyta (ferns)—Class Pteridopsida—Order Pteridales:

- Pteridaceae—Pteris semipinnata [8]

Divisions Pinophyta (conifers) and Ginkgophyta (Gymnospermae):

- Ginkgoaceae—*Ginkgo biloba* [15–20]
- Cupressaceae—*Juniperus communis* [21]
- Pinaceae—*Pseudolarix kaempferi* [22], *Picea glauca* [23]
- Taxaceae—*Taxus canadensis* [24–26], *Taxus cuspidata* [27,28], *Taxus chinensis* [26,27,29,30], *Taxus baccata* [25,31,32] and its varieties [28,33,34], *Taxus brevifolia* [28,35], *Taxus wallichiana* [36–38], *Taxus x Media* [26–28], *Taxus yunnanensis* [39]

Division Magnoliophyta (flowering plants; Angiospermae):
Class Magnoliopsida (dicotyledons):

- Asteraceae—*Stevia rebaudiana [40], Baccharis pingraea [41], Alomia myriadenia* [42]
- Apiaceae—*Anisotome* and other genera [43]
- Acanthaceae—*Andrographis paniculata* [44–47]
- Araliaceae—*Aralia* sp. [48]
- Ericaceae—*Rhododendron* sp. [49]
- Euphorbiaceae—*Croton tiglium* [50], *Euphorbia poisonii* [3], *Euphorbia leuconeura* [1,50,51], *Euphorbia lomii, E. pulcherrima, Codiaeum variegatum* [1], *Euphorbia lathyris* [2], *Clutia richardiana* [52], *Jatropha elliptica* [53]
- Celastraceae—*Tripterygium wilfordii* [54–56]
- Capparaceae—*Cleome viscosa* [57]
- Cistaceae—*Cistus creticus* subsp. *creticus* [58]
- Labiatae—*Teucrium chamaedrys* [4,59], *Ballota* sp. [5,60], *Teucrium fruticans* [61], *Salvia miltiorrhiza* [62–66], *Salvia fruticosa* [67], *Salvia divinorum* [68,69], *Sideritis* sp. [70], *Isodon rubescens (Rabdosia rubescens)* [71], *Rosmarinus officinalis* [72,73], *Ajuga remota* [6], *Lepechinia graveolens* [74], *Plectranthus* sp. [75]

- Fabaceae—*Caesalpinia bonduc* [76], *Hymenaea courbaril* var. *Stilbocarpa* [77], *Erythrophleum fordii* [78]
- Verbenaceae—*Vitex agnus-castus* [79], *Vitex rotundifolia* [80]
- Scrophulariaceae—*Scoparia dulcis* [81]
- Rubiaceae—*Coffea arabica* [82–84]
- Thymelaeaceae—*Wikstroemia monticola* [85], *Daphne genkwa* [86]
- Menispermaceae—*Tinospora sagittata, T. capillipes* [87]
- Meliaceae—*Azadirachta indica* [88,89]
- Salicaceae—*Salix matsudan* [90], *Casearia sylvestris* [91]
- Annonaceae—*Annona* sp. [92], *Annona glabra* [93], *Xylopia frutescens, Xylopia aromatica, Xylopia brasiliensis* [94]
- Pedaliaceae—*Harpagophytum procumbens* [95]

Class Liliopsida (monocotyledons):

- Orchidaceae—*Cymbidium* sp. [96]
- Potamogetonaceae—*Potamogeton lucens* [97]
- Poaceae—*Triticum* sp. [98] (gibberellins)
- Amaryllidaceae—*Polianthes tuberosa* [99] (gibberellins)
- Arecaceae—*Elaeis guineensis* [100] (gibberellins)
- Zingiberaceae—*Hedychium yunnanense* [101]

24.3.2 Marine Products

Diterpenes have been found in the following marine products:

- Brown algae (Phaeophyceae)—*Dictyota menstrualis* [102]
- Marine invertebrates—gorgonian octocorals (*Pseudopterogorgia elisabethae*, order: Gorgonacea) [103,104]; soft corals (*Sarcophyton* sp., order: Alcyonaria) [11]
- Marine sponges (*Rhopaloeides odorabile*, order: Dictyoceratida) [105]
- Mollusks—nudibranch (suborder Nudibranchia) [105]

24.3.3 Microorganisms (Including Fungi)

- Bacteria—*Proteus mirabilis, Proteus vulgaris, Klebsiella pneumoniae, Bacillus megaterium, Bacillus cereus, Escherichia coli* [106] (gibberellins)
- Lower fungi—*Gibberella fujikuroi* [107] (gibberellins)
- Class Basidiomycetes—*Hericium erinaceum* [108]

24.3.4 Insects

- Order Isoptera (termites)—Familia *Rhinotermitidae—Reticulitermes* sp. [109]

All of the mentioned species are widely discussed in this chapter.

24.4 BIOLOGICAL ACTIVITIES OF DITERPENES AND THEIR PHARMACOLOGICAL USE

Diterpenes supposedly play an important role in the defense system of plants [110]. For instance, diterpene dictyols produced by brown algae reduce their consumption by their predator, the marine amphipod [102]. *neo*-Clerodane diterpenes from *Ajuga remota* or *Teucrium fruticans* [6,61] as well

as beyerane and abietane diterpenes from *Plectranthus* species [75] display an insect antifeedant activity. Various diterpenes together with other compounds (e.g., mono- sesqui-, and sesterpenes) are known to be secreted by termite soldiers as their defensive substances [109]. Resin acids that occur in conifer oleoresins and mainly belong to the abietane and pimarane diterpenes play important roles in tree defense systems as insecticidal and antimicrobial agents [23]. Cembranoide diterpenes (sarcophine) are known to play a role in corals' chemical defense mechanism against natural predators [11]. Gibberellins are a group of diterpenes playing an important role in the physiology of plants as their hormones (e.g., germination, flowering stimulation).

In most cases, diterpenes and diterpene-containing plant materials exhibit pharmacological activity and are widely used in medicine. Tanshinones (diterpenes belonging to the abietane group), which are the main pharmacologically active compounds of *Salvia miltiorrhiza*, demonstrate antiplatelet aggregation, antioxidant, and other activities as well as a curative effect in cardiovascular diseases. *Salvia miltiorrhiza* is a traditional Chinese medicinal plant widely used in the treatment of many diseases [62,63,66]. *neo*-Clerodane compounds (mainly salvinorin C) occurring in *Salvia divinorum* are known for their psychotropic (hallucinogenic) properties [111].

Labdane diterpenes from Ladano resin (originating from *Cistus creticus* subsp. *creticus*) are known for their strong antibacterial properties [58]. It is generally confirmed that labdane diterpenes have antimicrobial, antiviral, anti-inflammatory, and cytotoxic activities [112]. Labdane diterpenes from *Alomia myriadenia* also show significant cytotoxic properties [42]. Andrographolides—labdane diterpenes from *Andrographis paniculata* leaves—are bitter compounds with immune-stimulating, anti-inflammatory, liver-protecting, bile-secreting, and fertility-decreasing properties [44,47].

Other labdane derivatives, namely, furanoid labdanes (e.g., saudin, richardianidin), are traditionally used as the main components of herbal infusions from *Clutia richardiana* and play the role of a hypoglycemic drug, which was confirmed in in vitro and in vivo experiments [52]. Some *ent*-kaurene derivatives demonstrate antibacterial, antifungal, and anti-inflammatory properties [36]; *ent*-kaurenes from the *Isodon* genus (Labiatae) indicate cytotoxic properties [58]. Other *ent*-kaurene compounds from the *Stevia* genus (stevioside, rebaudioside) are known for their sweetening purposes [40,113]. Other compounds of the *ent*-kaurene group—cafestol and kahweol (occurring in coffee beans)—also show anti-inflammatory and even anticarcinogenic properties. It is also confirmed that they elevate serum cholesterol levels [82,83].

Kaurenoic acid and other kaurenes are known for their anti-inflammatory, antimicrobial, and antiparasitical (against *Trypanosoma cruzi*) properties. For this reason, the materials from *Xylopia* species that contain these compounds are traditionally used as antirheumatic and antibacterial drugs [94].

Scopadulane-type tetracyclic diterpenes that occur in *Scoparia dulcis* (Scrophulariaceae), mainly as a scopadulcic acid, show antiulcer as well as antibacterial, antifungal, and even antitumor activities [81]. Trachylobanes are tetracyclic diterpenes that occur in the *Croton* species; they are reported to have cytotoxic properties [114]. Similar properties are found for bis-labdanic diterpenes from *Alpinia calcarata* [115]. Cembranoids occurring in the Capparaceae family (*Cleome viscosa* L.) demonstrate antibacterial as well as insecticidal and nematicidal properties [57]. Other cembrane diterpenes (sarcophine) occurring in soft corals are known for their cancer chemopreventive activity [11].

Triptolides, abietane-type diterpenes occurring in the *Tripterygium* genus (Celastraceae), are known as anti-inflammatory, immunosuppressive, antitumor, antioxidant, and antiviral compounds [54]. The *Tripterygium* species are widely used as anti-inflammatory plant materials in Chinese herbal medicine in the treatment of autoimmune disorders, such as rheumatoid arthritis, erythematosus, and skin diseases. Abietane diterpenes (e.g., carnosol or rosmanol) occurring in *Rosmarinus officinalis* or *Salvia fruticosa* exhibit strong activity as radical-scavenging antioxidants [67,72,73]. Abietane-type diterpenes from *Pseudolarix* species (e.g., *Pseudolarix kaempferi*) indicate antifungal as well as antitumor properties [22], while abietanes from *Salvia officinalis* reveal antiviral properties [116].

Clerodane diterpenes from *Casearia* species (Salicaceae)—casearins—show anti-ulcerogenic, antiseptic, and topical anaesthetic properties; they also have potent antitumour activity [91]. Pseudopterosins and seco-pseudopterosins, diterpene glycosides from gorgonian corals, are strong

anti-inflammatory and analgesic as well as cytotoxic agents [103,104]. Columbin is another example of an anti-inflammatory diterpene compound, as are furanoditerpenes from *Tinospora* species [87]. Meroditerpenoids from brown algae demonstrate strong cytotoxic properties [117].

Although plants from the family Euphorbiaceae are known from their toxic properties, some compounds from the clerodane group (occurring in *Croton oblongifolius*) have confirmed anti-cancer activities [118]. Anticancer properties (inhibitors of multidrug resistance in tumor cells) are also displayed by lathyrane-type macrocyclic diterpenes [119] and jatrophane diterpenes [53]. Other known cytotoxic compounds are *ent*-kaurenes (mainly oridonin) from *Isodon rubescens* (syn. *Rabdosia rubescens*), a Chinese traditional anticancer plant material [71].

Erinacines, which are unique diterpene xylosides found in *Basidiomycetes* fungi, have potent stimulating activity for nerve-growth-factor synthesis and agonistic activity for opioid receptors [108]. Taxanes are known for their antimitotic and cytotoxic properties, and they are used mainly in breast and ovarian cancer treatment (see paclitaxel [Taxol]). They fight cancer by promoting the creation of microtubules and inhibiting their depolimerization [32,120,121]. Ginkgolides are known for their antiplatelet properties and influence on the cerebral circulatory system. For these reasons, ginkgo leaves have been used to treat respiratiory and circulatory disorders for centuries [17,19].

Some diterpenes exhibit toxic (inflammation- and tumor-promoting) or skin-irritating activities. Such properties are reported for diterpenes from the Thymelaeaceae and Euphorbiaceae families, namely, the phorbol esters that belong to the tigliane, ingenane, and daphnane groups [3,85]. Tigliane, ingenane, lathyrane, and daphnane diterpenes from Euphorbiaceae exhibit both skin-irritant as well as tumor-promoting properties [1,2]. Grayanotoxins found in *Rhododendron* species (especially in their nectar) constitute another group of toxic diterpenes. These compounds are known for their neurotoxic properties, and they are responsible for honey intoxications in humans [49].

24.5 CHEMOTAXONOMIC IMPORTANCE OF DITERPENES

Diterpenes are a large and diversified group of compounds that are predominantly characteristic for systematic groups of plants and marine products. For instance, cembranes and prenylsesquiterpenes are important distinctive chemosystematic features for different populations of *Dictyota dichotoma*, a cosmopolitan species of marine brown algae [122]. Phorbol esters—belonging to the tigliane, daphnane, and ingenane diterpenes—have been found only in two plant families: Thymelaeaceae and Euphorbiaceae [3]. Spongiane diterpenes are characteristic structures for marine organisms—sponges [105].

Diterpenes from the kaurene group are characteristic for the *Aspilia* genus of the Asteraceae family [123]. Moreover, acyclic diterpenes found in the *Olearia* genus of the Asteraceae can be of chemotaxonomic importance for plants from the Astereae tribe [124]. Various groups of diterpenes occur only in some genera of the Asteraceae, and for this reason they are chemosystematically important in taxonomy of this family [125].

Chemotaxonomic differences exist between various species of the same genus; for example, *Wikstroemia canescens* contains a tigliane-type diterpene, whereas *Wikstroemia mekongenia* and *Wikstroemia monticola* contain daphnane-type diterpenes [85,126]. There are also differences in *Salvia* species: Only *Salvia divinorum* contains the *neo*-clerodane diterpene salvinorin, which is not found in *Salvia officinalis* [68]. Also, tanshinones are compounds characteristic only for *Salvia miltiorrhiza* [63]. Furtheremore, there are examples of chemotaxonomic variability even within the same species. Thus, differences in the pseudopterosin content of two chemotypes of soft coral *Pseudopterogorgia elisabethae* have been described [104].

Ginkgolides are a group of compounds occurring only in *Ginkgo biloba* (*Ginkgoaceae*) [17], whereas taxoids are a characteristic diterpene group occurring only in the *Taxus* genus [127]. However, there are differences in the content of selected taxanes, especially between individual species or subspecies of *Taxus*, which is of significant chemotaxonomical importance for this genus [127]. In contrast, gibberellins are plant hormones that are not specific to any plant systematic group. Therefore, they occur in both lower plants and fungi as well as higher plants [99,107,128]

24.6 HPLC AS A METHOD OF ANALYSIS OF DITERPENE COMPOUNDS IN NATURAL MATERIALS

HPLC (high performance liquid chromatography) is now a very popular and reliable technique for the qualitative and quantitative analysis of diterpenes. Many other separation techniques have also been applied in analysis of diterpenes, such as gas chromatography (GC) of some groups of diterpenes (more volatile ones, e.g., gibberellins) [99,100,107]; thin-layer chromatography (TLC) for quantitative analysis, fingerprint analysis, or process control of preparative chromatographic isolation of many groups of diterpenes (ginkgolides, taxanes, andrographolides, etc.) [28,32,47,120,129]; and electrochromatographic or electrophoretic methods—for example, micellar electrokinetic chromatography (MEKC)—applied for taxane quantitation [130]. In this last case, the MEKC technique seems to be better (economic, simple, fast) than the HPLC method [130].

24.6.1 Sample Pretreatment and Extraction and Purification Methods

In the HPLC analysis of secondary metabolites in plant materials, sample extraction and purification methods are very important and basic steps for a successful analysis; they influence the recovery and composition of analytes. Diterpenes are a miscellaneous group of compounds in the physicochemical and chromatographic sense, but they are generally of medium to low polarity. They often occur in plants in a glycoside form, and in this case, they are polar substances (soluble in water, methanol, etc.). This determines the choice of the extractant, which should have an affinity to the analytes and thus give exhaustive extraction. Generally, medium-polar solvents, such as dichloromethane [63,91], ethyl acetate [55], acetone [2,5], or even *n*-butanol [21], are used, but often strong polar solvents (e.g., methanol, mixtures of alcohols with water, or pure water) are used for the extraction of diterpenes [16,18,52,54,74].

In contrast, nonpolar solvents, such as *n*-hexane, are sometimes used for the extraction of low-polarity diterpenes (with other nonpolar, oily compounds), for example, for cafestol and kahweol, *ent*-kaurene diterpenes from coffee beans [82] or kaurenes from *Xylopia* species seeds [94]. Supercritical fluid extraction (SFE), which uses carbon dioxide as a solvent, is another kind of extraction applied to the diterpenes; in this case, the obtained oily fraction containing diterpenes is purified before quantitative HPLC analysis. Oil is saponified with potassium hydroxide in methanol, and the unsaponifiable fraction is then extracted with *n*-hexane and washed with water. After vaporization of *n*-hexane, the residue is dissolved in the mobile phase and quantified [82]. There are also examples of oil extracts containing diterpenes that require extraction with acetone [2]. Mixtures of dichloromethane and methanol are also used for the extraction of diterpenoids from marine organisms [11].

The purification of crude extracts is often necessary for adequate quantitative as well as qualitative analysis. It consists in removing ballast substances using liquid–liquid extraction (LLE), solid-phase extraction (SPE), and other techniques. In the analysis of labdane diterpenes in *Ballota* species, acetone extracts (after concentration) can be separated between ethyl acetate and water by use of LLE. In this step polar ballasts are removed with water. Thus, the organic phase may be evaporated, and the residue (after dissolution in the mobile phase) is ready for HPLC analysis [5]. After the evaporation of the solvent, crude oily extracts containing diterpenes (in acetone) can be purified using LLE with cyclohexane:acetonitrile (removing nonpolar ballasts with cyclohexane), followed by LLE with acetonitrile:petroleum ether (also removing nonpolar ballasts). Finally, the concentrated acetonitrile fraction, dissolved with methanol, after filtration can be exposed to acid or alkaline hydrolysis [2]. After such a procedure of purification, the HPLC analysis of diterpenes in *Euphorbia lathyris* oil is possible [2]. Extracts from corals (in methanol:dichloromethane) can also be purified using LLE, in this case with chloroform:water (the water phase removes sodium chloride residue); dichloromethane extract concentrated and recrystallized from cold *n*-hexane gives a purified diterpene fraction [11]. Another complicated sample-preparation procedure is used before

the analysis of plant hormones (e.g., gibberellins) in bacteria cultures. In this case, multistep LLE followed by preparative TLC is required [106].

In the analysis of taxanes in cell cultures or yew twigs, purification procedures including LLE and SPE can be usefull [24,131]. A methanolic extract of *Taxus* cell culture after evaporation is partitioned between water and dichloromethane, and then the organic fraction is purified on a Bond Elut NH_2 SPE cartridge. The fraction containing taxoids is than eluted with a mixture of methanol and dichloromethane, and the eluate is concentrated and dissolved in pure methanol [131]. A similar purification method consisting of extraction and SPE is used in the quantitative analysis of triptolide in plant material. In this case, we follow these steps: extraction with ethanol, concentration of the extract, dissolution in ethyl acetate, centrifugation and supernatant concentration, and finally extract (dissolved in dichloromethane:methanol [49:1]) purification on an SPE NH_2 cartridge. The obtained triptolide-containing fraction is evaporated, dissolved in ethanol, and analyzed by HPLC [54,56]. In the other cases, a Sep-Pak alumina B cartridge was used in SPE sample preparation of triptolide from a plant ethyl acetate extract [55]. An SPE technique on C-18 columns eluted with methanol:water (98:2) is used for the purification of concentrated crude dichloromethane extracts, which are subsequently dissolved in the eluent before the SPE purification [91]. In the quantitative analysis of jatrophone in *Jatropha elliptica* alcoholic extracts, only an SPE procedure on Supelclean LC-18 columns eluted with acetonitrile:water mixture is applied [53]. Another example of use of SPE for sample purification is the elution of kaurene (from *Xylopia* species seeds) with pure methanol from Adsorbex RP-18 cartridges (after *n*-hexane extraction, concentration, and residue dissolution in the methanol) [94]. There are also examples of SPE purification of taxanes on RP-2 columns eluted with methanol:water mixtures before quantitative analysis [34].

Sometimes, very complicated extraction and purification methods are used for the separation of diterpene compounds, in quantitative and even qualitative analysis. In the preparation of samples for quantitative HPLC analysis, quite a complicated purification procedure is sometimes necessary. For instance, the quantitation of diterpene esters in plants from the Euphorbiaceae requires the application of the following procedure: extraction of the plant material or natural latex using a methanol:water (17:3) mixture; LLE of extract obtained with *n*-hexane (to remove nonpolar ballasts); water addition into the extract to obtain methanol:water ratio of 1:2; and LLE of diterpene esters with diethyl ether. The diterpene esters obtained in this manner then undergo alkaline hydrolysis with 0.5 M methanolic potassium hydroxide. After neutralization with 0.5 M methanolic HCl, the obtained diterpene alcohols are fractionated by TLC on silica gel, using *n*-hexane:2-propanol (2:1, v/v) as a solvent system. The separated TLC fractions are scraped off, eluted with methanol, and finally destined for HPLC quantitative analysis [1].

The identification and simultaneous quantitation of ginkgolides also needs a sample-purification method. A water extract of *Ginkgo biloba* leaves is filtered through a celite, then the activated charcoal is added to adsorb the ginkgolides, the supernatant is centrifuged, and the compounds are desorbed with acetone. The extract prepared in this way is ready for further analysis [15]. Another purification method in the quantitative analysis of ginkgolides consists of methanol:water (1:4) or buffered water extraction, LLE with ethyl acetate, organic-phase concentration, and residue dissolution in methanol [16,17,20].

Also, in quantitative HPLC analysis of taxanes, a multistep sample-preparation procedure, including SPE on RP-8 or alumina columns followed by preparative TLC on silica, is necessary. In these cases, quantitative reversed-phase HPLC analysis of taxanes in an isocratic elution mode is possible [28,33]. A complicated sample-preparation procedure (based on LLE) before quantitative analysis of taxanes in plant material is described in yet another example [36]: A crude methanolic extract of needles of *Taxus wallichiana* is concentrated, stirred with water, and extracted with *n*-hexane (to remove nonpolar ballasts), then with chloroform; after that, the chloroform phase is concentrated, redissolved in methanol:chloroform (1:1), filtered through the Waters sample clarification kit, concentrated, dissolved in acetonitrile, and finally subjected to HPLC analysis [36]. Another procedure for sample preparation before taxane analysis is based on SPE purification. Crude extract (ethanol:water:acetic

acid, 80:19:1) is purified on Extrelut. The SPE column is first eluted with a mixture of petroleum ether:*t*-butyl methyl ether (8:2) to remove interfering compounds and then with pure *t*-butyl methyl ether to elute taxanes. The obtained fraction is concentrated and dissolved in methanol [31].

In the case of quantitation and identification of gibberellins in plant material, a complicated multistep purification and separation procedure is also necessary [99,128]. The procedure consists of methanol:water extraction; multistep LLE using water and organic solvents (hexane, ethyl acetate) at various pHs; SPE on QAE Sephadex anion-exchange columns; and finally multidimensional HPLC/GC separation [99] or LC-QTOF-MS/MS (quadrupole–time-of-flight tandem mass spectrometry) determination [128].

In contrast, simple extraction procedures, without any purification methods, are generally used in qualitative analysis (fingerprint, identification). For example, acetonitrile, water, or methanol extracts, after filtration, are directly used for HPLC analysis [72,74,80]. In such a case, the HPLC system is usually equipped with a precolumn cartridge to protect the analytical column from ballast substances [52,62,72,80]. Also, in the quantitative estimation of teucrine A in water infusions from *Teucrium chamaedrys* or beverages spiked with this herb, a simple sample preparation (filtration only) was sufficient; in this case, a precolumn is also used [4]. In the identification of salvinorin A in *Salvia divinorum* leaves, acetonitrile:water (1:1) extraction, filtration, and LC-MS analysis without even a precolumn cartridge was applied [68]. In the fingerprint analysis of tanshinones in *Salvia miltiorrhiza*, accelerated solvent extraction (ASE) with dichloromethane, concentration, and residue dissolution in methanol is used for sample preparation before HPLC separation [63].

In the quantitative analysis of andrographolides in plant material, the coupling of microwave-assisted extraction with HPLC allows the on-line determination of active compounds, which is important in fast, routine analysis. Dynamic microwave-assisted extraction (DMAE) using methanol:water mixtures coupled with HPLC analysis was optimized [44]. A microdialysis system coupled on-line with HPLC allowed the combination of a sample-preparation step with quantitative analysis of ginkgolides, which can be applied in rapid routine analysis of these metabolites in *Ginkgo biloba* leaves [18].

24.6.2 Identification of Diterpenes (Qualitative Analysis) in Natural Materials and Preparations

Qualitative HPLC analysis of diterpenes in natural samples is most often applied for identification or fingerprinting of plant materials (used, e.g., in the production of natural drugs). Figure 24.11 shows a fingerprint analysis of extracts from *Clutia richardiana* (Euphorbiaceae). Another important application of fingerprint analysis is chemotaxonomic analysis, which allows the discovery of chemotaxonomical markers describing differences between related taxonomic units (for example, fingerprint-based chemotaxonomy of *Taxus* species) [127].

The qualitative HPLC separation of tanshinones in *Salvia miltiorrhiza* followed by pharmacological activity profiling (MAO A and iNOS inhibitory effects) of effluent microfractions was elaborated, and in this way compounds of high pharmacological activity could be found [63]. Figure 24.12 shows activity profile for MAO A inhibitors in *Salvia miltiorrhiza* extract and corresponding HPLC fingerprint chromatogram [63].

There are also examples of HPLC application in the qualitative analysis of fractions separated using high-speed countercurrent chromatography (HSCCC). Cao et al. [29] shows HPLC analysis of taxane fractions (containing mainly 10-deacetylbaccatin III) isolated using HSCCC (see Figure 24.13). An analogous application of HPLC is a process control of HSCCC isolation of andrographolides from *Andrographis paniculata* leaves [46]. The analysis of the composition of fractions obtained by preparative vacuum liquid chromatography (VLC) or semipreparative HPLC is a similar application of analytical HPLC. The process of the isolation of *neo*-clerodane diterpenes [6,61] or daphnane diterpenes from *Wikstroemia monticola* [85] was controlled in systems using RP-18 columns with methanol + water. There are also examples of HPLC application in isolation, fraction control, analysis of isolated compounds, and elucidation of the chemical properties of pseudopterosins from gorgonian

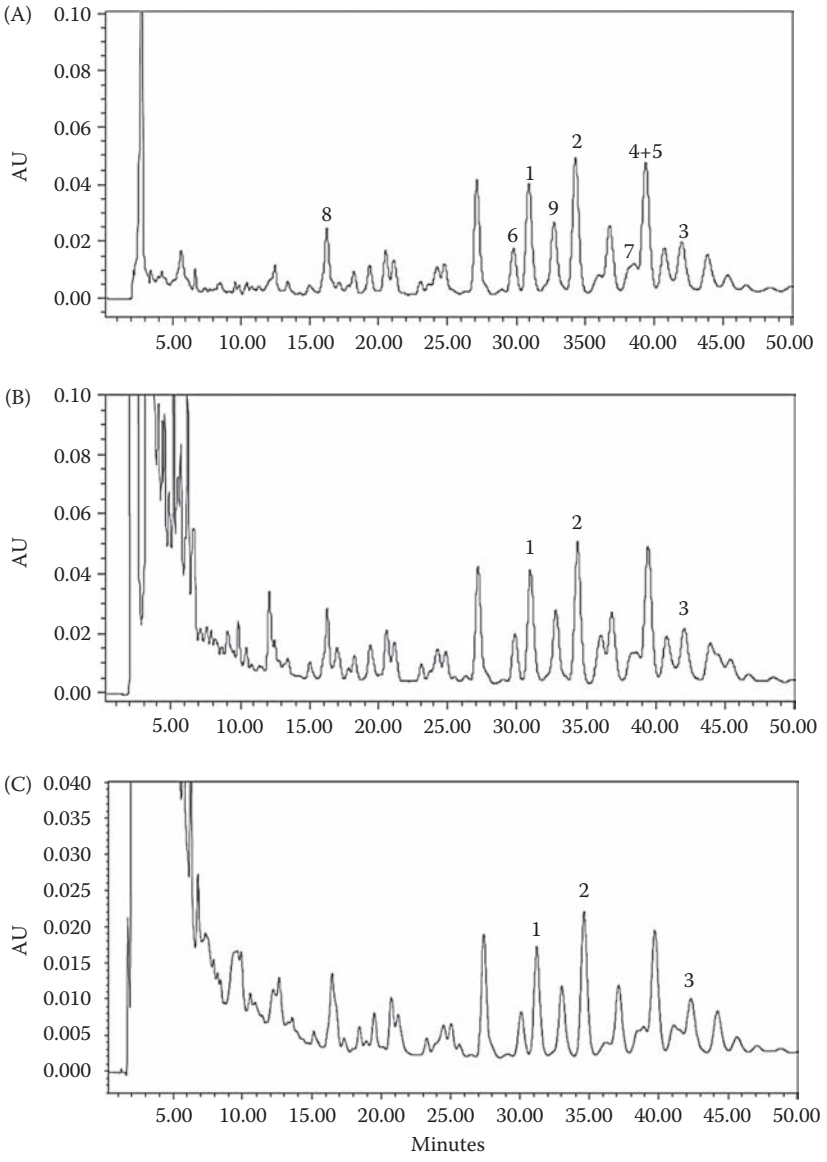

FIGURE 24.11 HPLC fingerprint analysis of *C. richardiana* obtained by extraction with (A) acetonitrile, (B) methanol, and (C) hot water. Peak assignment based on retention time matching and coinjection of pure markers: (1) Richardianidin-1; (2) Richardianidin-2; (3) Saudin; (4) Cluytene-A; (5) Cluytene-C; (6) Cluytene-B; (7) Cluytene-E; (8) Cluytene-F; (9) Saudinolide. (From Dimitrijević, S.M., Humer, U., Shehadch, M., Ryves, W.J., Hassan, N.M., and Evans, F.J., *J. Pharm. Biomed. Anal.*, 15, 393–401, 1996. With permission.)

corals [103]. An additional application of HPLC is composition control of fractions obtained from preparative supercritical fluid chromatography (prep. SFC) of rosemary extract [132].

24.6.2.1 Chromatographic Systems

Diterpenes are rather medium-polar compounds, so reversed-phase systems are most often used: RP-18 and RP-8 stationary phases and mixtures of polar organic solvents with water as eluents (see Table 24.1); UPLC columns are sometimes used [65,136]. Pentafluorophenyl- or phenyl-bonded silica columns eluted with acetonitrile:water mixtures [19,24,36] are other examples of reversed-phase

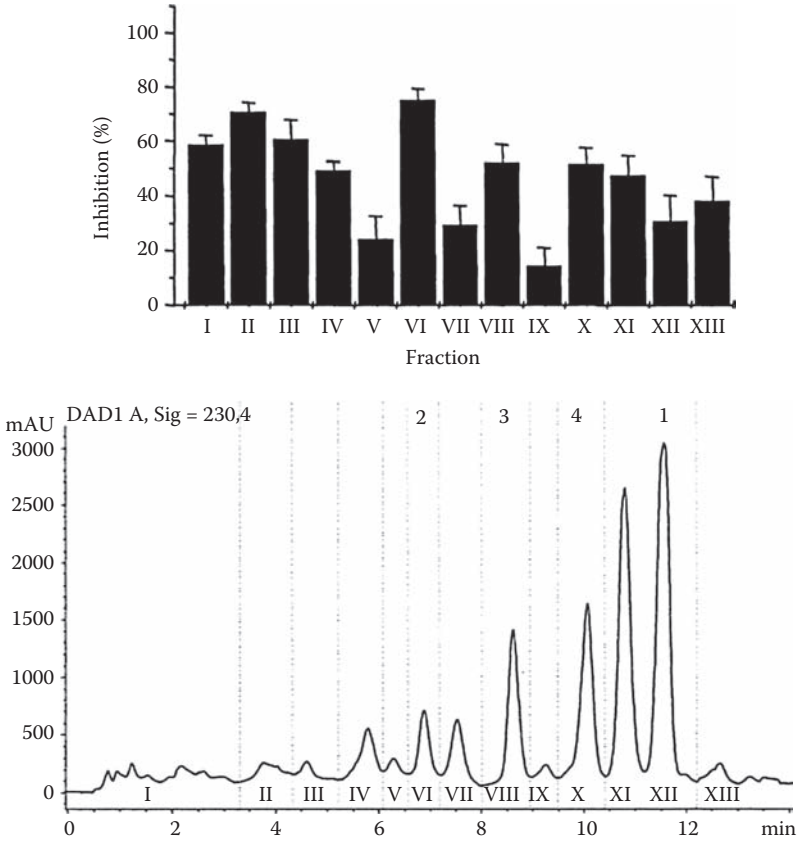

FIGURE 24.12 Activity profile for MAO A inhibitors in *S. miltiorrhiza* extract. The HPLC fingerprint of a dichloromethane extract recorded at 230 nm with time segments for microfractionation (I–XIII) is shown below, and the inhibitory activity of microfractions I–XIII is given above. For details see ref. (From Du, Q., Jerz, G., and Winterhalter P., *J. Chromatogr. A*, 984, 147–151, 2003. With permission.)

systems for the analysis of diterpenes. In the reversed-phase systems with MS detectors, similar chromatographic systems are used, but low flow rates of the mobile phase (e.g., 0.1–0.3 mL/min) are necessary [128] and/or eluate splitters are used—giving even 4 μL/min flow rates—for proper solvent evaporation before the analyte ionization (in the identification of taxanes by electrospray ionization [ESI]–MS) [131].

There are numerous examples of optimization of chromatographic conditions carried out on diterpene standards. Dolfinger and Locke [137] showed the results of optimization of chromatographic systems in the analysis of taxanes. They drew particular attention to the usefulness of various fluorinated stationary phases (linear perfluorohexyl, branched propyl perfluorophenyl, perfluorophenyl) and a C-8 RP stationary phase, eluted with gradient of acetonitrile in water [137]. These optimized chromatographic systems can be used in the analysis of diterpenes in natural mixtures. There are also examples of optimization of HPLC separation of natural compounds performed not only on standards mixtures but additionally on plant extract samples, for example, the optimization of HPLC separation of some taxanes from *Taxus* species extracts performed using the computer program DryLab G [121]. There are also examples of SFC separation of diterpenes (taxanes) on a Diol column eluted using carbon dioxide:methanol [135].

Other examples present normal-phase separation on a cyano-bonded column eluted with hexane:methanol [5] or a silica gel column eluted with chloroform:methanol [3] or 0.8% of 2-propanol in hexane [84].

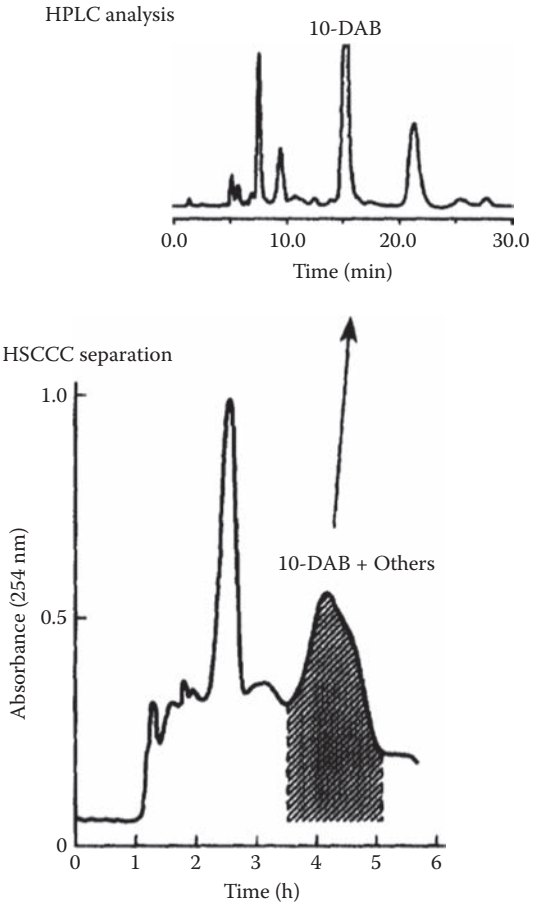

FIGURE 24.13 The first-step high-speed countercurrent chromatography (HSCCC) separation of the crude needle extract of *Taxus chinensis* and HPLC analysis of the 10-deacetylbaccatin III (10-DAB) fraction. For experimental conditions see ref. (From Martin, A.M., Queiroz, E.F., Marston, A., and Hostettmann, K., *Phytochem. Anal.*, 17, 32–35, 2006. With permission.)

24.6.2.2 Identification Methods (Detection Modes) and Coupled Techniques in Separation of Diterpenes

Absorption of UV light is a phenomenon that is broadly used in the detection and identification of diterpenes. Although their UV spectra are rather nonspecific and have maxima in the low wavelength values ($\lambda = 190–230$ nm) [6,91], UV detectors or diode array detectors (DADs) are still the most popular and accessible in the HPLC kits. In the case of UV detection problems, the application of an evaporative light scattering detector (ELSD) [6,61] or refractive index (RI) detector [17] is a solution. Another interesting method of detection applied in the analysis of diterpenes is ELISA, which is quite complicated (time-consuming, and one has to have appropriate antibodies for the compounds analyzed) but highly selective and drastically lowers the limits of detection and quantitation. Therefore, the ELISA method can successfully be used for trace analysis in complicated natural matrices. ELISA analysis of gibberellins (besides with cytokinins) in oil palm is an example of the application of this method [100].

Coupled techniques in HPLC analysis relate to identification and detection problems. The most widespread "coupled technique" is the connection of HPLC separation with spectral methods (MS, rarely nuclear magnetic resonance [NMR]). The main advantage of such a connection is the

TABLE 24.1
Qualitative HPLC Analysis (Identification) of Diterpenes

Compounds	Sample	Stationary Phase/Column	Mobile Phase	Detection	Ref.
Ginkgolides	*Ginkgo biloba* — leaves	C-18 column, Microsorb 3 Spherical, ODS2 (3 µm, 100 × 4.6 mm) preceded by ODS2 guard column	Methanol:water, gradient elution	SSI–ion trap MS identification; RI quantitation	[17]
ent-Labdanes	*Potamogeton lucens* — herb	Zorbax Eclipse XDB-Phenyl columns (5 µm, 250 × 4.6 mm) -two columns connected in series	Acetonitrile:water:tetrahydrofuran, gradient elution	DAD – 210 and 254 nm; APCI–MS/MS	[97]
Furanoid labdanes — richardianidin, richardianidin-2, saudin, saudinolide, cluytenes	*Clutia richardiana* — herb (methanolic extract)	Pre-packed Luna C18 column (5µm, 250 × 4.6 mm) preceded by SecurityGuard cartridge system (Phenomenex)	A – water; B – acetonitrile:tetrahydrofuran: methyl-*tert*-butyl ether (96:2:2); linear gradient from 30 to 35% B, during 45 min	UV – 220 nm; DAD identification	[52]
Furanoid labdanes	*Baccharis pingraea* — compounds isolated by prep. HPLC	Alltech Econosil Silica gel column (5 µm, 250 × 4.6 mm)	20% ethyl acetate and 0.1% formic acid in hexane	APCI–MS	[41]
Abietanes (e.g., rosmanol, carnosol) simultaneously with phenolics	*Rosmarinus officinalis* — leaves; methanolic extract	endcapped Ultrabase C18 (5 µm, 250 × 4.6 mm) preceded by C18 guard column	A – acetonitrile; B – water + 0.1% H_3PO_4; various linear gradients	DAD	[72]
			A – acetonitrile; B – water + formic acid; linear gradient	ESI–ion trap MS	
Abietanes (e.g., rosmanol, carnosol) simultaneously with phenolics	*Rosmarinus officinalis* — leaves; fractions from prep. SFC	Nova Pack C-18 column (3.5 µm, 150 × 4.6 mm)	A – 1% acetic acid in acetonitrile; B – 1% acetic acid in water; gradient elution	DAD – 230 nm	[132]
Abietanes (e.g., rosmanol, carnosol) simultaneously with phenolics	*Lepechinia graveolens* — herb	Luna C18 column (5 µm, 150 × 2.1 mm)	A – acetonitrile; B – 0.1% formic acid in water; linear gradient	DAD – 280 and 365 nm; ESI–MS/MS identification	[74]

Abietanes (e.g., rosmanol, carnosol)	*Salvia fruticosa* — herb; infusions	Kromasil C-18 column (5 µm, 250 × 4.6 mm)	A – acetonitrile; B – water + 0.1% H_3PO_4; various linear gradients	UV – 230 nm	[67]
Tanshinones	*Salvia miltiorrhiza* — root	LiChrospher 100 RP-18 (5 µm, 125 × 4.0 mm)	A – methanol; B – water; linear gradient	DAD – 230 nm; API–MS	[63]
Tanshinones (abietanes) simultaneously with phenolics	*Salvia miltiorrhiza* — rhizome + root	Zorbax Extend C18 column (5 µm, 250 × 4.6 mm) + Zorbax C18 guard column	A – water + formic acid; B – acetonitrile; linear gradient	UV – 281 nm; ESI–TOF-MS	[62]
Tanshinones (abietanes)	*Salvia miltiorrhiza* — root	Alltech, Alltima C18 column (5 µm, 250 × 4.6 mm)	Acetonitrile:water:formic acid, gradient elution	DAD – 280 nm	[133]
Tanshinones (abietanes)	*Salvia miltiorrhiza* — rhizome + root	Zorbax SB-C18 column (5 µm, 250 × 4.6 mm)	A – acetonitrile; B – 0.5% acetic acid in water; C – methanol; gradient elution	DAD – 280 nm; ESI–MS	[66]
Salvinorin A	*Salvia divinorum* — leaves	Luna C18 column (3 µm, 150 × 2mm)	A – acetonitrile; B – water + formic acid; linear gradient	DAD; ESI–ion trap MS	[68]
Salvinorin A	*Salvia divinorum* — leaves	UPLC BEH C18 column (1.7 µm, 100 × 2.1 mm)	Acetonitrile:water, gradient elution	ESI–TOF-MS	[69]
(+) and (–) Isoabienol	*Jungermannia infusca* — isolated compounds	Chiralcel OD-H chiral column	*n*-Hexane:2-propanol (9:1), isocratic elution	—	[14]
Beyeranes, abietanes	*Plectranthus* sp. — leaves	LiChrospher 100 C-18 column (5 µm, 250 × 4 mm)	A – methanol:water:acetic acid (90:5:5); B – 2% acetic acid in water; linear gradient	DAD	[75]
Labdanes — hispanolone, ballonigrine, dehydrohispanolone	*Ballota* sp.	Zorbax–CN (5 µm, 250 × 4.6 mm)	Hexane:methanol (98:2, v/v), isocratic elution, flow rate gradient	DAD identification (UV spectra 190–360 nm)	[5,60]
Labdanes (rotundifuran) simultaneusly with other groups of compounds	*Vitex rotundifolia* — fruits	Zorbax SB-C18 column (3.5 µm, 250 × 4.6 mm) + Zorbax C18 guard column	A – acetonitrile; B – water + formic acid; gradient elution	DAD – 300 nm	[80]

(*Continued*)

TABLE 24.1 (CONTINUED)
Qualitative HPLC Analysis (Identification) of Diterpenes

Compounds	Sample	Stationary Phase/Column	Mobile Phase	Detection	Ref.
Labdanes	*Juniperus communis* — berries	Nova-Pak C-18 column (5 µm, 250 × 4.6 mm)	A – methanol; B – water + trifluoroacetic acid (0.05%); gradient elution	DAD – 210 and 254 nm	[21]
Labdanes	*Alomia myriadenia* — herb; isolated fractions	Shimpak prep C-18 column (250 × 4.6 mm)	Acetonitrile:water, isocratic elution	UV – 254 nm	[42]
Andrographolides	HSCCC fractions: *Andrographis paniculata* — leaves	Ultrasphere C-18 column (5 µm, 150 × 4.6 mm)	Methanol:water:acetonitrile (5:5:1), isocratic elution	UV – 230 nm	[46]
neo-Clerodanes	*Ajuga remota [6]* or Teucrium fruticans [61] — preparative VLC and HPLC fractions	Lichrospher 100 RP-18 column (5 µm) + C18 guard column	Methanol:water, gradient elution	DAD – 205 nm; ELSD	[6,61]
ent-Kaurenes (e.g., oridonin) simultaneously with flavonoids	*Isodon rubescens* — herb	Zorbax–Extend C-18 column (5 µm, 250 × 4.6 mm)	Methanol:water, gradient elution	DAD – 242 nm; ESI–ion trap MS	[71]
Chinane diterpenes	*Harpagophytum procumbens* — roots	C18(2) column Phenomenex Luna (3 µm, 150 × 4.6 mm); postcolumn: Poly-(divinylbenzene) SPE cartridges	A – acetonitrile; B – water; linear gradient	DAD – 230 nm; NMR (600 MHz) identification	[95]
Phorbol esters — ingenanes, daphnanes, tiglianes	*Euphorbia poisonii* — preparative TLC and HPLC fractions	Apex SI — silica column (5 µm, 250 × 4.6 mm)	A – $CHCl_3$ (99%); B – methanol (1%)	DAD – various wavelengths	[3]
Lathyranes, ingenanes	*Euphorbia lathyris* — seed oil	HP C-18 Hypersil column (5 µm, 200 × 4.6 mm) + guard column	A – acetonitrile:water (1:1); B – acetonitrile	DAD – 204 and 280 nm	[2]
		Nucleosil 100 C18 AB (5 µm, 125 × 2 mm)	A – water + formic acid; B – acetonitrile + formic acid; linear gradient	ESI–MS	
Ingenanes and tiglianes	*Euphorbia leuconeura* — latex; *Croton tiglium* — oil	Supersphere C-18 column (4 µm, 250 × 4 mm)	Water:acetonitrile, gradient elution	UV– 220 nm; turbo ion spray–MS/MS identification	[50]

Daphnanes	*Daphne genkwa* — bark, buds, roots	Shimadzu C-18 column (5 µm, 150 × 4.6 mm)	Methanol:water (85:15), isocratic elution	UV – 232 nm	[86]
		Zorbax C-18 column (5 µm, 150 × 2.1 mm)	Methanol:water (85:15), isocratic elution	API–ESI MS	
Taxanes	*Taxus* sp. — cell cultures	Michrom microbore HPLC system — C-18 Vydac column (5 µm, 150 × 1 mm)	Water:methanol:acetonitrile mixtures, linear gradient	ESI–MS/MS	[131]
Taxanes	HSCCC fractions: *Taxus chinensis* — needles	Microsob-MV - C-8 column (5 µm, 150 × 4.6 mm)	Acetonitrile:water (25:75), isocratic elution	UV – 230 nm	[29]
Taxanes	*Taxus baccata var. Aurea* — preparative column fractions	Symmetry C-18 column (3.5 µm, 150 × 4.6 mm)	Acetonitrile:water, gradient elution	DAD – 233 and 280 nm	[134]
Taxanes	*Taxus* sp. — needles	Kromasil C-18 column (5 µm, 200 × 4.6 mm)	Acetonitrile:water, gradient elution	DAD – 227 nm; ESCi–MS	[127]
Taxanes	*Taxus wallichiana* — needles [37], bark [38]	Spherisorb ODS-2 column (5 µm, 250 × 4.6 mm)	Acetonitrile:1 mM ammonium acetate in water:methanol (2:2:1), isocratic elution	DAD – 280 nm; ESI–MS/MS	[37,38]
Taxanes	*Taxus* sp. — needles	LiChrospher 100 RP-18 column (5 µm, 250 × 4.6 mm)	A – deuterated water + 0.1% trifluoroacetic acid; B – acetonitrile + 0.1% trifluoroacetic acid; gradient elution	NMR (LC–NMR in stopped-flow mode)	[26]
Taxanes — paclitaxel, cephalo-mannine	*Taxus brevifolia* — needles	Microcolumn (1 m × 0.250 mm) packed with Spherisorb 5 µm ODS-2 stationary phase	Methanol:water:acetonitrile (3:3:4), isocratic elution	UV – 225 nm	[35]
Taxanes	*Taxus yunnanensis* — wood and bark	XTerra MS C-18 column (5 µm, 150 × 2.1 mm)	A – methanol; B – water + acetic acid (0.5%); linear gradient	ESI–FT-MS (IonSpec QTF-7 quadrupole Fourier-transform ion cyclotron resonance hybrid mass spectrometer)	[39]

(*Continued*)

TABLE 24.1 (CONTINUED)
Qualitative HPLC Analysis (Identification) of Diterpenes

Compounds	Sample	Stationary Phase/Column	Mobile Phase	Detection	Ref.
Taxanes — paclitaxel and related compounds	Taxanes — standards mixture	Supercritical fluid chromatography — LiChrospher diol column (5 µm, 250 × 4.6 mm)	Carbon dioxide:methanol; 150 bar pressure, gradient elution	UV – 227 nm	[135]
Taxanes + other compounds	*Taxus* sp. — hair roots	UPLC BEH C18 column (1.7 µm, 100 × 2.1 mm)	Acetonitrile:water, gradient elution	TUV detector – 227 nm; ESI–MS	[136]
Cassane butenolide diterpenes	Compounds isolated from *Caesalpinia bonduc*	Hypersil Gold (20 × 4 mm) guard column	A – 0.1 mM ammonium acetate in acetonitrile; B – 0.1 mM ammonium acetate in water (7:3); isocratic elution	ESI–MS/MS identification	[76]
Cassaine diterpenes	*Erythrophle-um fordii* — bark, isolated fractions	Agilent XDB-C18 column (5 µm, 150 × 2.1 mm)	Acetonitrile:water, various proportions	ESI–MS/MS identification	[78]
			Acetonitrile:deuterated water, various proportions	NMR identification, structure elucidation	
		Agilent XDB-C18 column (5 µm, 150 × 4.6 mm)	Acetonitrile:water, various proportions	ESI–TOF-MS accurate mass measurment	
Diterpene xylosides — erinacines	*Hericium erinaceum* — thallus — isolated fractions	Cretspak C-18 S column (150 × 4.6 mm)	Methanol:water (8:2) or acetonitrile:water (4:7), isocratic elution	UV – 210 nm	[108]
Anisotomene-type irregular diterpenes	*Anisotome* sp. — herbs	Zorbax Rx-C18 column (3.5 µm, 150 × 4.6 mm) + guard column LiChrospher C-18	A – 0.15% acetic acid in water; B – 0.15% acetic acid in methanol; linear gradient	DAD – 205 nm; APCI–MS identification	[43]

Note: APCI, atmospheric-pressure chemical ionization; DAD, diode-array detection; ELSD, evaporative light-scattering detector; ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; RI, refractive index (detection); SSI, sonic spray ionization; TOF, time of flight; VLC, vacuum liquid chromatography. API MS, Atmospheric Pressure Ionization Mass Spectrometry; API ESI MS, Atmospheric Pressure Ionization Electro Spray Mass Spectrometry; ESI MS, Electro Spray Mass Spectrometry; ESI–FT-MS (IonSpec QTF-7 quadrupole Fourier-transform ion cyclotron resonance hybrid mass spectrometer); TUV, Tunable Ultraviolet Detector.

improvement of identification of compounds in complicated mixtures: without the need of time-consuming isolation procedures and in low concentrations (when we do not have enough sample), even if we do not obtain sufficient separation of compounds analyzed. Obviously, LC-MS and LC-NMR have some technical disadvantages (time-consumption, costs, problems related to use of several eluents, such as evaporation, or potential ionization of analytes), but they are still indispensable in the search of new compounds, mainly pharmacologically active metabolites. For example, for the identification of ginkgolides using LC–sonic spray ionization (SSI), ion trap MS was applied [17]. ESI coupled with time-of-flight (TOF)–MS or ion trap MS is more popular in the identification of natural compounds and broadly applied in diterpene analysis [37,62,68,72]. Figure 24.14 shows an example of HPLC–ESI–ion trap MS analysis of diterpenes and flavonoids in an herb of *Isodon rubescens*, compared with the corresponding HPLC-DAD analysis [71].

There are also examples of application of coupled ultra performance liquid chromatography (UPLC)–ESI–MS techniques used for the identification of metabolites in plants—for example, the identification of 47 taxanes from other compounds as metabolic profiling of *Taxus* hair roots [136].

There are many applications of LC-NMR in the analysis of natural mixtures (including diterpenes). For the identification of major substances in simple mixtures, the on-flow techniques are more efficient. For detailed structure elucidation of the major peaks by means of 2D-NMR or 1D-NMR of minor peaks, stopped-flow and loop storage methods are necessary. When the concentration of the analyte is limited, sample-manipulation techniques such as SPE, multiple trapping, and the use of a cryogenic probe are appropriate. When the sample amount is limited, capillary liquid

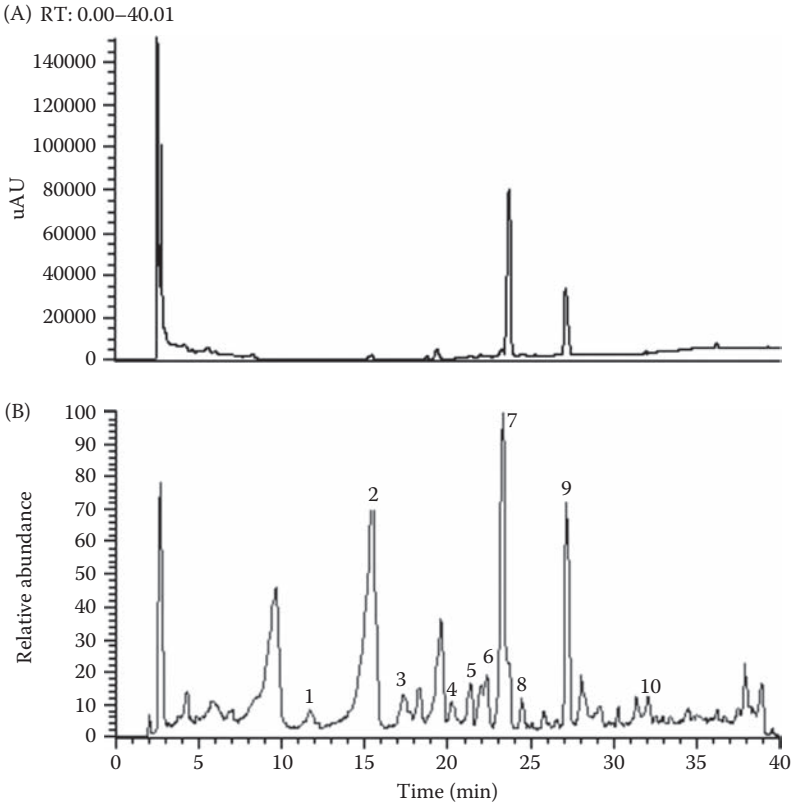

FIGURE 24.14 HPLC–electrospray ionization–tandem mass spectrometry analysis of the extract of *Isodon rubescens*. (A) HPLC–UV chromatogram monitored at 242 nm; (B) liquid chromatography–negative-ion electrospray ionization–mass spectrometry total ion current (TIC) profile. (From Williams, L.A.D., Vasques, E., Reid, W., Porter, R., and Kraus, W., *Naturwissenschaften*, 90, 468–472, 2003. With permission.)

chromatography (capLC)–NMR is the method of choice. The ongoing development of LC-NMR holds much promise for progress in both the field of plant metabolomics and the analysis of natural products [138]. There are also examples of LC-NMR separation and identification of new compounds in plant material. For instance, on-line hyphenated HPLC-SPE-NMR made it possible to identify new chinane diterpenes in crude extracts of *Harpagophytum procumbens* roots [95] and enabled the analysis of abietanes (e.g., rosmanol, carnosol) in *Rosmarinus officinalis* [138] and separation and identification of taxanes in *Taxus* species needles [26].

Another task for coupled techniques is rapid and reproducible quantitative analysis of metabolites (diterpenes among others) in natural materials. There are many examples of on-line coupling of sample-extraction techniques (dynamic microwave-assisted extraction) or sample-preparation techniques (SPE, microdialysis) with HPLC analysis [18,44,139].

24.6.3 Multidimensional HPLC Separation of Diterpenes

In the separation of complicated natural mixtures, high-tech chromatographic methods are indispensable. One of a few possibilities is the use of various chromatographic techniques combined in a one analytical run—multidimensional chromatography. This technique can be composed of a few HPLC systems of various selectivity or HPLC combined with another separation method (GC, TLC, electromigration methods). One example of multidimensional HPLC of diterpenes is the separation and identification of gibberellins in plant material (corms of *Polianthes tuberosa*). After a complicated sample-preparation procedure—LLE and SPE (see Section 24.6.1)—gibberellins are separated on an HPLC C-18 Bondapak column and a Nucleosil C-2 column. Next, the fractions obtained are identified by dwarf rice bioassay, and finally the bioactive fractions are derivatized (methylated and trimethylsilylated) and analyzed using capillary GC-MS [99].

Another example of HPLC-GC analysis of a diterpene compound (simultaneously with sterols) is 16-*O*-methylcafestol determination in green coffee beans. In this case, rapid authenticity analysis is performed in the following system: HPLC (Hypersil silica gel column [5 μm, 100×2.1 mm] eluted with 0.8% 2-propanol in *n*-hexane, with UV detection at 205 nm) followed by backflushing of the column with *tert*-butyl methyl ether, and finally capillary GC-MS analysis [84]. In another experiment, semipreparative HPLC separation (in various normal- and reversed-phase systems on silica, NH_2, and C-18 columns) followed by capillary GC-MS analysis was used for the separation and identification of clerodane diterpenes in *Hymenaea courbaril* var. *Stilbocarpa* seed pod resin [77]. Diterpenes (geranyl linalool, geranyl geraniol, geranyl geranial) occurring in termites' defense secretions were separated and analyzed, simultaneously with other terpene groups, using HPLC-GC-MS. Finally, HPLC-separated fractions were identified using NMR [109].

24.6.4 Quantitative Analysis of Diterpenes

Simplicity and reproducibility are the most important features in quantitative analysis. Therefore, simple sample-preparation techniques coupled on-line with HPLC analysis can be the best solution in routine quantitative analysis. For example, a dynamic microwave-assisted extraction coupled with reversed-phase HPLC enables the rapid determination of andrographolides in plant material [44], and a microdialysis system coupled with HPLC gives good results in sample purification and simultaneous quantitation of ginkgolides in ginkgo leaves [18].

HPLC with UV (DAD) detection is the conventional standard method in the quantitative analysis of various diterpene compounds. In many cases, diterpenes have no good UV detection, which results in relatively high limits of detection and quantitation. Sometimes there can be a real problem, especially when diterpenes occur in plant materials in very low concentrations (for example, taxanes, ginkgolides, and others) in the presence of comparatively high concentrations of compounds from other groups (e.g., flavonoids, polyphenols, etc.). In such cases, a very complicated purification as well as preliminary separation procedures seem to be indispensable [24,54,129].

Also, the optimization of the conditions for an analysis is very important and helps determine the best separation of compounds intended for quantitative analysis. Bala et al. described the detailed optimization of the separation of taxanes [36]. The authors optimized the extraction and purification procedures as well as the conditions for the analysis: Four HPLC columns (C-18, phenyl, or pentafluorophenyl) and three ternary gradient systems (water, acetonitrile, and methanol) were examined. The best resolution for taxanes was achieved using the Nova-Pak Phenyl column [36]. Sample preparation and purification is the next factor having a tremendous influence on the quantitative determination of plant metabolites (e.g., diterpenes). Numerous papers describing the optimization of the drying and storing of plant material, of extraction yields, and finally of the purification of extracts before a proper analysis. The preceding steps seem, however, to be more important in the analysis of thermolabile and chemolabile compounds. ElSohly et al. in their paper described the optimization of drying conditions for plant material (*Taxus* species needles) and their influence on the quantity of taxanes in the investigated samples [25].

Sometimes, if we analyze a group of compounds with similar properties (e.g., similar UV spectra) and want to estimate the yield of extraction and purification for the whole group, we can employ the response surface methodology (the total surface of all analyzed peaks in HPLC chromatogram is compared for samples prepared in different ways). An example of such an analysis is the optimization of conditions for the extraction of casearins from *Casearia sylvestris*. In this analysis several solvents, degrees of acidity, extraction modes, and extraction times as well as sample drying and storage conditions were compared [91]. Another possibility is to employ other detection methods in quantitative analysis, such as MS [129], RI [20], or ELSD [4,92,129,140]. Croom et al. applied ELSD in the determination of total terpene lactones in *Ginkgo biloba* materials and preparations [140].

There are also examples of chromatographic methods being replaced with other techniques, one of which is matrix-assisted laser desorption ionization (MALDI) connected with MS. A good example of the application of this technique is preliminary quantitative analysis of taxanes in a cell culture medium, tentatively developed for standards of taxanes. Executed SPE-MALDI-MS experiments show that this technique could be used for rapid screening of taxane molecules in *Taxus* cell cultures over a large number of samples, and a preliminary quantitation can also be achieved. In the comparison with this method, LC-MS analysis is considered to be more time consuming and therefore can be performed on only the more promising or more complicated samples [141].

The majority of quantitative analyses relate to quality assessment of plant material. This concerns all plant materials used in natural drug production [45]; isolation of pharmacologically active compounds; food production, for example, as flavorings (there are examples of the determination of natural diterpene sweeteners in beverages [113]) or food supplements (e.g., *Ginkgo biloba* leaves [142]). There are also numerous examples of quantitative analysis of diterpenes in pharmaceutical preparations or semiproducts (extracts, oils, etc.) [20]. Figure 24.15 shows an example of quantitative analysis (internal standard mode) of labdane diterpenes in a drug sample from the fruit of *Vitex agnus-castus* [79].

The process control in extraction, separation, or isolation procedures (e.g., qualitative and quantitative HPLC of HSCCC-isolated taxane fractions [29] or SFE-process quantitative HPLC assessment in extracts of *Rosmarinus officinalis* or *Ginkgo biloba* [19,73]) is another application of quantitative HPLC analysis of diterpenes. There are also examples of quantitative HPLC analysis application in process control of preparative chromatography (column chromatography or preparative TLC)—for example, isolation of taxanes from plant material [32,120].

There are also numerous examples of quantitative HPLC determination of diterpenes in animal or medical samples—urine, plasma, tissues, etc. [139,143]. Since this book focuses on other topics, this field of analysis has been treated marginally in this chapter. Figure 24.16 shows an example of HPLC analysis of andrographolides in rat urine after oral administration of extract from *Andrographis paniculata* leaves [143].

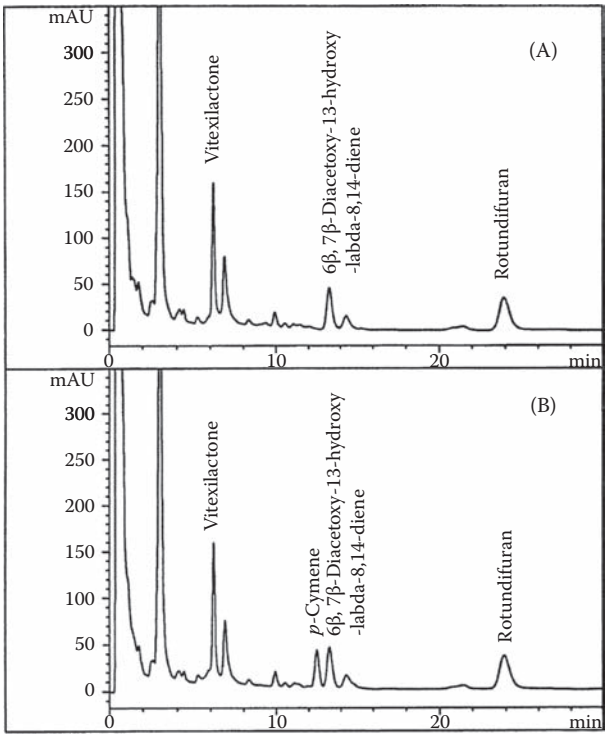

FIGURE 24.15 Chromatogram of a drug sample of Agni-casti fructus without (A) and with (B) internal standard *p*-cymene. (From Yilmaz, B.S. and Çitoglu, G.S., *Fabad J. Pharm. Sci*., 28, 13–17, 2003. With permission.)

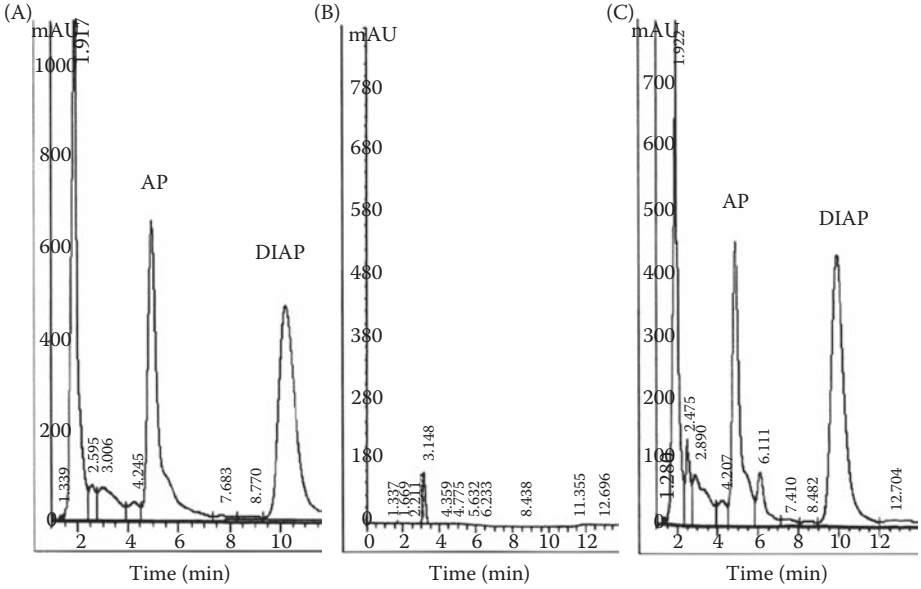

FIGURE 24.16 (A) HPLC of methanol extract of *Andrographis paniculata*; (B) HPLC of rat urine before oral administration of extract; (C) HPLC of pooled rat urine obtained within 24 h after an oral dose of 1 g/kg *A. paniculata* extract. Column: LiChrosorb RP-18 (250 × 4.6 ID, 10 μm); flow rate: 1 mL/min; detection: 210 nm. AP, andrographolide; DIAP, 14-deoxy-11,12-dideoxyandrographolide. (From Bosisio, E., Giavarini, F., Dell'Agli, M., Gali G., and Gali, C.L., *Food Addit. Contam*., 21, 407–414, 2004. With permission.)

TABLE 24.2
Quantitative HPLC Analysis of Diterpenes

Compounds	Sample	Stationary Phase/Column	Mobile Phase	Detection	Ref.
Ginkgolides	*Ginkgo biloba* — leaves; phar-maceuticals	μ-Bondapack C18 column (10 μm, 300 × 3.9 mm)	Water:methanol (67:33, v/v), isocratic elution	UV – 220 nm	[15]
Ginkgolides	*Ginkgo biloba* — leaf extract	C-18 column	Water:methanol (77:23, v/v), isocratic elution	UV	[16]
Ginkgolides	*Ginkgo biloba* — leaves	Zorbax SB-C18 column (5 μm, 150 × 4.6 mm) preceded by microdialysis system	Methanol:acetonitrile:0.01 M phosphate buffer (30:5:65, v/v/v), isocratic elution	UV– 219 nm	[18]
Ginkgolides	*Ginkgo biloba* — leaves and preparations	C-18 column — Microsorb 3 Spherical, ODS2 (3 μm, 100 × 4.6 mm) preceded by ODS2 guard column	Methanol:water (29.5:70.5, v/v), isocratic elution	RI quantitation; SSI–ion trap MS identification	[20]
Ginkgolides	SFE extracts from leaves of *Ginkgo biloba*	Pentafluorophenyl Curosil-PFP column (5 μm, 150 × 4.6 mm)	Water:acetonitrile, gradient elution	DAD + ESI–ion trap MS	[19]
Ingenanes and tiglianes	leaves or latex from: *Euphorbia* sp. and *Codiaeum variegatum*	Spherisorb RP-18 column (2.5 μm, 250 × 4.6 mm)	Water:acetonitrile, linear gradient from 0 to 88% acetonitrile in 40 min	UV– 220 nm + identification by MS	[1]
Ingenol, phorbol, 12-deoxyphorbol	*Euphorbia leuconeura* — leaves and latex	Spherisorb RP-18 column (5 μm, 250 × 4.6 mm)	Water:acetonitrile, gradient elution	UV– 280 nm	[51]
Jatrophone	*Jatropha elliptica* — underground stems	Shim-pack CLC-C-18 column (5 μm, 250 × 4.6 mm) + C-18 precolumn	Water:acetonitrile, gradient elution	UV– 280 nm	[53]
Andrographolides	*Andrographis paniculata* — leaves	Pinnacle 11 C18 column (5 μm, 250 × 4.6 mm)	65% methanol in water	DAD – 225 nm	[44]
Andrographolides	*Andrographis paniculata* — leaves; phar-maceutical preparations	Symmetry column C18 (5 μm, 150 × 3.9 mm)	A – 0.1% formic acid in water; B – acetonitrile; gradient elution	DAD – various wavelengths; ESI–MS	[45]
Andrographolides	*Andrographis paniculata* — leaves	μ Bondapak C-18 column (10 μm, 300 × 3.9 mm)	Acetonitrile:water (7:3, v/v), isocratic elution	UV– 230 nm	[47]

(*Continued*)

TABLE 24.2 (CONTINUED)
Quantitative HPLC Analysis of Diterpenes

Compounds	Sample	Stationary Phase/Column	Mobile Phase	Detection	Ref.
Andrographolides	Rat urine after oral administration of *Andrographis paniculata* leaf extract	LiChrosorb RP-18 (10 µm, 250 × 4.6 mm)	Methanol:water (6:4, v/v), isocratic elution	UV– 210 nm	[143]
Andrographolides	Rabbit plasma after oral administration of *Andrographis paniculata* leaf extract	Pinnacle 11 C18 column (5 µm, 250 × 4.6 mm) + C-18 guard column as SPE	Methanol:acetonitrile:water (5:1:4), isocratic elution	UV – 225 nm	[139]
Columbin	Rat plasma after oral administration of columbin from *Tinospora* sp.	Luna C-18 column (5 µm, 100 × 2 mm) + SB C-18 guard column	A – methanol; B – water + ammonium acetate; isocratic elution	electrospray –MS/MS	[87]
Kaurane, atisane, labdane, and other diterpenes	*Sideritis* sp. — herb	Hypersil C-18 column (5 µm, 150 × 4.6 mm)	Methanol:water (7:3, v/v), isocratic elution	DAD – 220 nm	[70]
Labdanes — vitexilactone, rotundifuran, etc.	*Vitex agnus-castus* — fruits; pharmaceutical preparation	Hypersil C-18 column (5 µm, 125 × 3.1 mm)	Acetonitrile:water, gradient elution	DAD – 210 nm	[79]
Labdanes — hedychenone	*Hedychium yunnanense* — herb	C-18 column	Acetonitrile:water (9:1), isocratic elution	UV – 235 nm	[101]
Kaurenes — kaurenoic acid, 16-α-hydroxy- kauranoic acid, xylopic acid	*Xylopia* species — seeds, leaves, and stems	ODS C18 column (5 µm, 250 × 4.0 mm)	70% acetonitrile in water	UV – 220 nm	[94]
Kaurenes — e.g., kaurenoic acid	*Annona glabra* — bark	Rainin C-18 column (5 µm, 250 × 4.6 mm)	Acetonitrile:water (85:15), isocratic elution	DAD – 210 nm	[93]
Cafestol, kahweol — *ent*-kaurenes	*Coffea arabica* — green and roasted beans	HP-C18 (5 µm, 200 × 4.6 mm)	Methanol:water (85:15, v/v), isocratic elution	UV – 220 nm	[82]
Cafestol, kahweol — *ent*-kaurenes	*Coffea arabica* — coffee brews	Superspher Lichrocart 100 C-18 column (3 µm, 250 × 4.6 mm)	Methanol:water, gradient elution	UV – 230 and 290 nm	[83]
ent-Kaurenes	*Annona* sp.	Zorbax 80A Extend - C18 column	Acetonitrile:water + acetic acid, gradient elution	ELSD	[92]

ent-Kaurenes — stevioside, rebaudioside	*Stevia rebaudiana* — leaves	NH_2 – LiChrospher or NH_2 – Zorbax columns	acetonitrile-water isocratic or gradient elution	UV or DAD – 210 nm	[40]
ent-Kaurenes — stevioside, rebaudioside and derivatives	Beverages with natural sweeteners	Synergi-Hydro column (4 µm, 250 × 4.6 mm) + Phenomenex Security Guard C_{18} cartridge	A – NH_4OAc/HOAc buffer in water; B – acetonitrile; C – acetic acid in water; gradient elution	UV – 215 nm; CAD impurities identification	[113]
ent-Kaurenes	*Pteris semipinnata*	Diamonsil ODS (5 µm, 150 × 4.6 mm)	A – acetonitrile (30%) B – 2 mmol.L^{-1} ammonium acetate in water (70%)	APCI–quadrupole MS	[8]
Scopadulanes — scopadulcic acid A and B, scoparic acid, scopadulin	*Scoparia dulcis* — leaves	Cromosil C-18 -AR packed column (5 µm, 150 × 4.6 mm)	Methanol:0.01 M H_3PO_4 in water (3:1, v/v), isocratic elution	UV – 230 nm	[81]
neo-Clerodanes	*Teucrium chamaedrys*	Phenomenex Luna C18 (5 µm, 150 × 4.6 mm)	Acetonitrile:water, gradient elution	DAD – 220 nm	[59]
neo-Clerodanes — teucrin A	*Teucrium chamaedrys* — herb infusions and beverages	Adsorbosphere C-18 (5 µm, 250 × 4.6 mm)	Acetonitrile:water, gradient elution	UV– 220 nm + ESI–ion trap MS identification	[4]
Taxanes	*Taxus canadensis* — needles, twigs	Supelcosil LC-F pentafluorophenyl endcapped column (5 µm, 250 × 4.6 mm) + Curosil G guard column	Acetonitrile:water, gradient elution	UV– 227 nm + DAD identification	[24]
Taxanes	*Taxus wallichiana* — needles	Nova-Pak Phenyl (4 µm, 150 × 3.9 mm)	Water:acetonitrile:methanol, gradient elution	DAD – 228 nm	[36]
Taxanes — paclitaxel, 10-DAB III	*Taxus baccata* — needles	Alltech Nucleosil C-18 (5 µm, 250 × 3.2 mm)	Acetonitrile:water + ortho-phosphoric acid, gradient elution	DAD – 227 and 280 nm	[31]
Taxanes	*Taxus cuspidata*, *T. chinensis*, *T. x Media* — needles	Phenomenex Curosil (penta-fluorophenyl) column (5 µm, 250 × 4.6 mm)	Acetonitrile:water, gradient elution	UV – 234 nm	[27]
Taxanes — paclitaxel	*Taxus chinensis* — cell cultures	Alltech Econosil C-18 column (5 µm, 250 × 4.6 mm)	Acetonitrile:water, gradient elution	DAD – 227 nm	[30]
Taxanes — paclitaxel, 10-DAB III	*Taxus baccata* var. *Aurea* — twigs	Symmetry C-18 column (5 µm, 250 × 4.6 mm)	Acetonitrile:water (3:7 for 10-deacetylbaccatin III; 1:1 for paclitaxel), isocratic elution	DAD – 200 and 230 nm	[33]

(*Continued*)

TABLE 24.2 (CONTINUED)
Quantitative HPLC Analysis of Diterpenes

Compounds	Sample	Stationary Phase/Column	Mobile Phase	Detection	Ref.
Taxanes — paclitaxel, 10-DAB III	*Taxus* sp. — needles, twigs	Adsorbosphere C-18 column (5 µm, 250 × 4.6 mm)	Acetonitrile:water (3:7 for 10-deacetylbaccatin III; 6:4 for paclitaxel), isocratic elution	UV – 232 nm	[28]
Taxanes — 10-DAB III, cephalomannine, paclitaxel	*Taxus baccata* — twigs; fractions after preparative column and thin-layer chromatography	Symmetry Shield C-18 column (5 µm, 150 × 4.6 mm)	Acetonitrile:water, gradient elution	DAD – 230 nm	[32,120]
Taxanes	*Taxus baccata* var. *Aurea, T. baccata* var. *Elegantissima*	Symmetry C-18 column (3.5 µm, 150 × 4.6 mm)	Acetonitrile:water, gradient elution	DAD – 233 and 280 nm	[34]
Casearins — clerodanes	*Casearia sylvestris* — leaves	Supelcosil C-18 (5 µm, 250 × 4.6 mm)	Acetonitrile:water, gradient elution	DAD – 235 nm	[91]
Sarcophine — cembranolides	*Sarcophyton* sp. — marine animal material (coral)	TSK-Gel C-18 - 80TM column (5 µm, 150 × 4.6 mm)	Acetonitrile:water + phosphoric acid, isocratic elution	UV – 220 nm	[11]
Pseudopterosins	*Pseudopterogorgia elisabethae* — marine animal material (soft coral)	Dynamax C-18 column (5 µm, 250 × 5 mm)	Water:acetonitrile, gradient elution	UV – 210 nm	[104]
Spongianes	*Rhopaloeides odorabile* —marine animal material (sponge)	Alltech C-18 column (5 µm, 250 × 4.6 mm)	Methanol:water (81:19), isocratic elution	UV – 230 nm	[105]
Anisotomene-type irregular diterpenes	*Anisotome* sp. — herbs	Zorbax Rx-C18 column (3.5 µm, 150 × 4.6 mm) + guard column Phenomenex C-18	A: 0.01% trifluoroacetic acid in water; B: acetonitrile; linear gradient	UV– 205 nm; interial standard quantitation	[43]

Triptolide — abietanes	*Tripterygium wilfordii* — roots	Phenomenex Curosil (pentafluorophenyl) column (5 µm, 250 × 4.6 mm) + phenylpropyl guard cartridge	Acetonitrile:water, gradient elution Acetonitrile:methanol:water, isocratic elution	DAD – 219 nm DAD – 219 nm	[54] [56]
Triptolide, tripdiolide	*Tripterygium wilfordii*	Nova-Pak C18 column	Acetonitrile:water (19:81 for triptolide; 11:89 for tripdiolide), isocratic elution	UV – 214 nm	[55]
Tanshinones (abietanes)	*Salvia miltiorrhiza* — root	Alltech, Alltima C18 column (5 µm, 150 × 2.1 mm)	A – acetonitrile (70%); and B – formic acid (1.6%) in water (30%), isocratic elution	ESI–MS/MS	[64]
Tanshinones (abietanes)	*Salvia miltiorrhiza* — root	UPLC BEH C18 column (1.7 µm, 50 × 2.1 mm)	Acetonitrile:water, gradient elution	DAD – 270 nm	[65]
Abietanes (e.g., abietic acid)	*Picea glauca* — phloem	Alltima C18 column (5 µm, 250 × 4.6 mm)	methanol:5% acetic acid:water (85:5:10), isocratic elution	DAD – various wavelengths (various λ_{max} for all determined compounds)	[23]
Abietanes —abietic acid, dehydroabietic acid	Colophony (resin from *Pinaceae* family) containing medications	PartiSphere C-18 column (5 µm, 110 × 4.6 mm)	A – methanol (87%) and B – phosphoric acid (0.02%) in water (13%), isocratic elution	DAD – 200 and 239 nm; fluorimetric – 225 nm (excitation) and 285 nm (emission)	[144]
Abietanes (e.g., pseudolaric acid)	*Pseudolarix kaempferi* — bark	Inertsil ODS-3 column (5 µm, 250 × 4.6 mm)	Methanol:0.5% acetic acid in water, gradient elution	DAD – 262 nm	[22]
Abietanes (e.g., rosmanol, carnosol) simultaneously with phenolics	SFE extracts from leaves of *Rosmarinus officinalis*	Nova-Pak P C18 (4 µm, 150 × 3.9 mm)	A – water + acetic acid; B – acetonitrile + acetic acid; gradient elution	DAD –230 nm + ESI–MS identification	[73]
Pimarane diterpene simultaneously with triterpenoids	*Aralia* sp. — herbs	Supelco SIL-LC-18 column	Methanol:1% formic acid in water:tetrahydrofuran (500:110:1, pH 3.4), isocratic elution	UV – 205 nm	[48]
Gibberellic acid + other plant hormones	*Triticum* sp. — seeds	HiQ Sil C-18 column (5 µm, 250 × 4.6 mm)	A – 0.2% formic acid in water and B – methanol (1:1), isocratic elution	ESI–ion trap MS identification + quantitation	[98]
Gibberellins simultaneously with other plant hormones	*Macadamia integrifolia*	Alltech LCMS C-18 column (3 µm, 20 × 2.1 mm) + Phenomenex C-18 guard column	A – 0.02% acetic acid in water B – methanol:acetonitrile (80:20)	QTOF-MS/MS identification + quantitation	[128]

(*Continued*)

TABLE 24.2 (CONTINUED)
Quantitative HPLC Analysis of Diterpenes

Compounds	Sample	Stationary Phase/Column	Mobile Phase	Detection	Ref.
Gibberellins simultaneously with other plant hormones	Bacteria cultures — various species	Supelcosil LC-18 column (5 µm, 250 × 4.6 mm)	A – phosphoric acid in water and B – methanol (7:3), isocratic elution	UV detection – 208 nm	[106]
Gibberellins besides cytokinins	*Elaeis guineensis*	Spherisorb 5 C-18 column (5 µm, 250 × 4.9 mm)	A – acetic acid (2 mM) in water; B – methanol; linear gradient	ELISA method using special immunoassay plates coated with antibodies; UV – 254 nm control	[100]

Note: APCI, atmospheric pressure chemical ionization; CAD, charged aerosol detector; DAD, diode array detector; ELSD, evaporative light scattering detector; ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem mass SPECTROMETRY; NMR, nuclear magnetic resonance; RI, refractive index (detection); SSI, sonic spray ionization; QTOF, quadrople time of flight.

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25 High Performance Liquid Chromatography of Triterpenes (Including Saponins)

Wieslaw Oleszek and Anna Stochmal

CONTENTS

25.1 INTRODUCTION

Triterpenes are a large group of naturally occurring substances with relatively complex cyclic structures consisting of a carbon skeleton based on six isoprene units. They are formed by "head-to-head" condensation of two farnesyl pyrophosphate units. The product of this reaction is an acyclic C_{30} hydrocarbon, squalene. The squalene can be formed both in plant and in animal organisms and plays a major role in the biosynthesis of 30-carbon triterpenes as well as steroids, which play many physiological functions in the synthesizing organisms. The cyclization of squalene leads to the formation of pentacyclic structures of oleane, ursane, and lupine type. The hydroxyl derivatives of these types are known under their trivial name, saponins. Their glycosylated forms can be found in many plant families.

Tetracyclic triterpenes occur more often in living organisms than pentacyclic triterpenes. This group has been represented by gossypol isolated from cotton and by cucurbitacines, the toxic and bitter principles of some Cucurbitaceae. Closely related to cucurbitacines are tetracyclic triterpenes, which are steroids that result from squalene cyclization into lanosterol in animal organisms and into cycloartenol in plants. These compounds have a large structural diversity due to the number of substitutions of the functional groups in the steroid skeleton. Besides, they may occur in glycosidic forms, known under their trivial name as steroidal saponins, glycoalkaloids, and cardiac glycosides.

Both pentacyclic and tetracyclic triterpenes and their glycosides are biologically active substances, and their determination in plant material, pharmaceutical formulas, foods, and feed additives requires development of analytical methods for their determination. One of the most reliable method for this their determination is high performance liquid chromatography (HPLC). Many analytical protocols and registration techniques have been worked out. The triterpenes lack chromophores that would allow UV detection, so that their HPLC analysis remains a challenge.

25.2 HPLC COLUMNS AND SOLVENT SYSTEMS

HPLC is the most powerful and the most frequently used technique for triterpene and steroidal determination because it can deal effectively with nonvolatile, highly polar compounds. It has been used extensively for determination of both aglycones and intact saponins. The separations are usually performed on normal- (silica gel) and reversed-phase (C8, C18) columns, of which C18 is definitely preferred, but other modified silica gel supports including NH_2 and DIOL are occasionally used (Table 25.1).

In some instances, when resolution of saponins on reversed-phase columns is insufficient, selected carbohydrate, borate anion-exchange, and hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] supports have been used successfully. Carbohydrate and NH_2-modified columns have been shown to be very effective in the separation of glycoalkaloids [55], but some steroidal saponins were also successfully analyzed [119]. β-Chaconine, α-chaconine, and α-solanine were nicely separated with a μ-Bondapack NH_2 column in the reversed-phase mode in less than 7 min (detection limit 5–15 ppm) [53]. Borate anion-exchange chromatography depends on the formation of borate complexes with *cis*-diols in the saccharide moiety. The formation of these complexes in some cases significantly improves the resolution and separation of isomeric glycosides that are not separated on a reversed-phase support [43]. After separation, genuine saponins can be recovered by removing borate as volatile methyl borate by repeated codistillation of the eluate with methanol. Resolution of closely related compounds can also be improved by the application of a hydroxyapatite support. The hydroxyapatite is more hydrophilic than a silica gel support and allows separation of two glycosides differing only in a terminal pentose [44].

25.3 DETECTION

25.3.1 UV Detection

The main problem in HPLC analysis of triterpenes is detection. Only a few triterpenes have absorption maxima in the UV range, and these can be easily detected at 254 nm. They include saponins that are constituents of licorice (*Glychyrrhiza glabra*): glycyrrhizin, glycyrrhetinic acid, and sweet saponin (Figure 25.1) [134,135].

Chemically, glycyrrhizin is a sulfated polysaccharide. It is considered the active constituent of the drug, and the standardization of licorice is based on its glycyrrhizin content. These compounds possess a conjugated diene function and are responsive to monitoring by UV detection at 254 nm; a detection limit of 0.125 μg/mL has been reported for these compounds.

The second group of compounds that can be monitored with UV detectors are the bitter principles of some Cucurbitaceae fruits, called cucurbitacines (Figure 25.2). The absorption maximum for these compounds is at 232 nm. For 2,3-dihydro-2,5-dihydroxy-6-methyl-4-pyrone (DDMP) conjugated soyasaponins, which have an UV absorption maximum at 295 nm, routine UV detection can also be successfully applied. Similar absorption has been shown by avenacins occurring in oat (*Avena sativa*) roots (Figure 25.3).

The majority of triterpenes have none of the chromophores necessary for UV detection, and the separation of intact glycosides or their aglycones has to be traced at lower (200–210 nm), rather nonspecific wavelengths. But at this wavelength other components of the analyte than saponin may

TABLE 25.1
HPLC Determination of Saponins

Sample	Column	Solvent System	Ref.
Aescin	C18	MeCN–H_2O	[1]
Acutangulosides A–F	RP-18, phenyl	MeOH–H_2O	[2]
Aesculiside A	ODS YGW C18	CH_3CN–H_2O–OHAc	[3]
Aesculus sap.	C18	MeCN–H_2O–H_3PO_4	[4]
	C18	MeCN–H_2O–H_3PO_4	[5]
Agave sapogen.	C8	MeCN–H_2O (benzoate esters)	[6]
Albiziasaponins A–E	YMC-Pack ODS-A	CH_3CN–H_2O–OHAc	[7]
Amaranthus sap.	C18	MeCN–H_2O (Br derivatives)	[8]
Aster sap.	C18 Nova-Pak	MeCN–H_2O	[9]
Asterosaponins	RP-18	MeOH–H_2O	[10]
Avenacosides	RP8	MeCN–H_2O	[11]
Avicins D and G	Intersil RP-18	MeOH–H_2O	[12]
	Flurosep-RP-phenyl	CH_3CN–H_2O	
Barringtogenol C	C-18 201SP510	CH_3CN–H_2O–TFA	[13]
Barringtonia sap	YMC-Pack ODS-AQ	MeOH–H_2O	[14]
Boswellic acid	RP-18	CH_3CN	[15]
Calendula sap.	C18	MeOH–H_3PO_4	[16]
Capsicosides E–G	μ-Bondapack C-18	MeOH–H_2O	[17]
Celtis triterpenes	YMC J'sphere ODS-H80	CH_3CN–H_2O	[18]
Chenopodium sap.	μ-Bondapack C-18	MeOH–H_2O	[19]
Certonardosides A–J	YMC-Pack ODS	MeOH–H_2O	[20]
	YMC-Pak ODS	MeOH–H_2O	[21]
	C18-5E Shodex	MeOH–H_2O	
	YMS-Pak C-8	MeOH–H_2O	
Conyzasaponins I–Q	Pegasil ODS-II	MeOH–H_2O	[22]
		CH_3CN–H_2O	
Cucumariosides	Silasorb C-18	CH_3COCH_3–H_2O	[23]
Cucurbitacins	C18	MeOH–H_2O (45:55 or 70:30)	[24]
Dioscorea sap.	Cosmogel C-18	MeOH–H_2O	[25]
Diosgegnin acet.	Silica gel	Hexane–*iso*PrOH	[26]
Echinocystic acid glc.	ODS	CH_3CN–H_2O–TFA	[27]
Elburzensosides	μ-Bondapack C-18	MeOH–H_2O	[28]
Ficus triterp.	Silica gel	EtOAc–hexane	[29]
Flos sap.	Zorbax SB-C18	CH_3CN–H_2O–OHAc	[30]
Ginsenoside Rb1	Lichrosorb-NH2	MeCN–H_2O–BuOH (8:2:1)	[31]
Ginsenosides	Silica gel LS-310	*n*-C_6H_{14}–CH_2Cl_2–MeCN (15:3:2) Benzoyl	[32]
	Silica gel	*n*-Heptane–BuOH–MeCN–H_2O	[33]
	Silica gel	CH_3Cl–MeOH–H_2O (30:17:2)	[34]
	Spherical silica gel	Hexane–Et_2O–EtOAc	[35]
	Bondapack C18	MeCN–H_2O (1:1–9:1)	[36]
	C18	MeOH–H_2O	[33]
	C18	MeCN–H_2O	[37]
	C18	MeOH–H_2O	[38]
	C18	MeCN–50 nM KH_2PO_4	[39]

(Continued)

TABLE 25.1 (CONTINUED)
HPLC Determination of Saponins

Sample	Column	Solvent System	Ref.
		$MeCN–H_2O–H_3PO_4$	
	C18	$MeCN–H_2O–H_3PO_4$	[40]
	C18	$MeCN–H_2O$	[41]
	C18	$MeCN–H_2O$	[42]
	Anion exchange	$MeCN–0.25\ M\ H_3BO_3$ (12.5:87.5)	[43]
	Hydroxyapatite	$MeCN–H_2O$ (80:20)	[44]
		$MeCN–H_2O$ (90:10 → 70:30)	
Ginsenosides	YMC-Pack ODS-AQ	$CH_3CN–H_2O$	[45]
	YMC-Pack ODS-AQ303	CH_3CN–10 mM K-phosph. buffer	[46]
Glinus sap.	RP-18	$CH_3CN–H_2O$	[47]
Hederagenin sap.	Sigel	$CHCl_3$–MeOH	[48]
	YMC R&D ODS	$MeOH–H_2O$–TFA	[49]
Helleborus sap.	µ-Bondapack C-18	$MeOH–H_2O$	[50]
Hydrocotylosides I–VII	ODS	$CH_3CN–H_2O$	[51]
	Capcell Pak Ph	$CH_3CN–H_2O$–TFA	
Hydroxyimberic acid sap.	PrepLC	$CH_3CN–H_2O$–TFA	[52]
Glycoalkaloids	µBondapack NH_2	$THF–H_2O–MeCN$ (56:14:30)	[53,54]
	NH_2	$MeCN–KH_2PO_4–H_2O$	[55]
	Carbohydrate	$MeCN–THF–H_2O$	[56]
	C18	$MeOH–H_2O–H_3PO_4$	[57]
	C18	MOH–0.01 M Tris, MeCN–0.01 M Tris	[58]
	C18	$MeCN–H_2O$–ethanolamine (45:55:0.1)	[59]
	YMC-Pack SiO2	$CHCl_3$–MeOH–NH_4OH (10:13:1; 7:13:2)	[60]
Glycyrrizin	C18	$MeOH–H_2O$–TBA–H_3PO_4	[61]
	C18	$MeOH–H_2O$–OHAc	[62]
	C18	$MeOH–H_2O–HClO_4$	[63]
	C18	MeCN–MeOH–H_2O + ammonium perchlor.	[64]
Gypenosides	C18	$MeOH–H_2O$	[65]
Gyspogenin glcAC18	$MeOH–H_2O$–TBA–H_3PO_4	[66]	
H. helix sap.	C18	$MeCN–H_2O$	[67]
Ilex sap.	µ-Bondapack C-18	$MeOH–H_2O$	[68]
Jenisseensosides A–C	Lichrospher RP-18	$CH_3CN–H_2O$–TFA	[69]
Jujubogenins	µ-Bondapack RP-18	$MeOH–H_2O$	[70]
Justicosides A–D	YMC ODS	$CH_3CN–H_2O$	[71]
Lotoidesides A–F	X-Terra C-18	$MeOH–H_2O$–TFA	[72]
Lycianosides A–C	µ-Bondapack C-18	$MeOH–H_2O$	[73]
Lupanes	Kromasil Sil	cyclohexane–EtOAc	[74]
Lupinus sap.	Reliasil C-18	$CH_3CN–H_2O$–TFA	[75]
Maesa sap.	Hypersil BDS C18	$NH_4OAc–MeOH–CH_3CN–H_2O$	[76]
Medicagenic acid sap.	XTerra RP-18	$CH_3CN–MeOH–H_2O$	[77]
Medicagenic acid	C18 Hypersil	$MeOH–H_2O$–HCOOH	[78]
Medicagenic acid glc	Eurospher C18	$H_2O–MeCN$ (10–90%) Br derivatives	[79,80]
Medicago sap.	RP-18	$CH_3CN–H_2O$–OHAc	[81]
	RP-18	$CH_3CN–H_2O$–OHAc	[82]
Mimengosides C–G	Pegasil OGS-II	$MeOH–H_2O$	[83]

TABLE 25.1 (CONTINUED)
HPLC Determination of Saponins

Sample	Column	Solvent System	Ref.
Morolic acid	RP-18 201SP	CH_3CN–H_2O–TFA	[84]
Nephelioside I–VI	ODS-AQ	MeOH–H_2O	[85]
Nerium sap.	C18 Nova Pak	MeCN–H_2O–TFA (20:80 → 45:55)	[9]
Notoginsenosides	YMC-Pak ODS-A	CH_3CN–H_2O–OHAc	[86]
Oenothera triterpenoids	Lichrosorb Diol	*n*-Hexane–ethyl acetate	[87]
Oleanolic acid	C18	MeCN–H_2O	[88]
Oleanolic acid sap.	Develosil PhA	CH_3CN–H_2O–TFA	[89]
	RP-18 201SP	CH_3CN–H_2O–TFA	[84]
	YMC R&D ODS	MeOH–H_2O–TFA	[27]
Oleane glc.	μ-Bondapak C18	H_2O–MeCN (70:30 → 50:50)	[90]
		H_2O–MeCN (40–70%) Br derivatives	[90]
	RP-8, DIOL	H_2O–MeCN (85:15)	[91]
Pachyelasides A–D	Asahipack GS-320	MeOH	[92]
Panax sap.	Synergi Hydro-RP	CH_3CN–H_2O	[93]
Phytolaccagenic acid glc.	STR Prep-ODS 20	MeOH–H_2O	[94]
Pittoviridoside	YMC ODS-A	MeOH–tetrahydrofuran–H_2O–OHAc	[95]
Primula sap.	C18	MeCN–H_2O–H_3PO_4	[96]
Protoaescigenin sap.	C18 201SP510	CH_3CN–H_2O–TFA	[13]
Protobassic acid sap.	ODS Zorbax	CH_3CN–H_2O	[97]
Pulsatilla sap.	Spherisorb ODS 2	CH_3CN–H_2O	[98]
Quinonemethide triter.	RP-18	MeOH–H_2O–H_3PO_4	[99]
Saikosaponin	Aquasil, hydroxyapatite	H_2O–MeCN, $CHCl_3$–MeOH–H_2O	[100]
	Develosil-ODS	MeOH–H_2O	[101]
	Nucleosil 50-5	$CHCl_3$–MeOH–EtOH–H_2O (62:16:16:6)	[34]
	Silica gel	$CHCl_3$–MeOH–H_2O (30:10:1)	[34]
	C18	MeCN–H_2O	[101]
	C18	MeOH–H_2O–OHAc–trimethylamine	[102]
Saniculasaponins I–XI	ODS	CH_3CN–H_2O	[103]
Scabiosaponins A–K	Pegasil ODS	MeOH–H_2O	[104]
Serianic acid sap.	STR Prep-ODS 20	MeOH–H_2O	[94]
Soyasapogenol A, B	ODS RP-18	CH_3CN–PrOH–H_2O–OHAc	[105]
Soyasaponins	Lichroprep RP-18	CH_3CN–H_2O–TFA	[106]
	μ-Bondapack RP-18	MeOH–isoPrOH–H_2O–HCOOH	[107]
	Zorbax Eclipse XDB-C18	CH_3CN–H_2O	[108]
	Aquasil RP-18	CH_3CN–H_2O–OHAc	[109]
Soyasapogenols	Silica gel	Petrol–EtOH	[110]
Soyasaponins	Lichrosorb RP18	MeOH–PrOH–H_2O–OHAc (32:4:63:0.1)	[111]
	Silica gel	$CHCl_3$–MeOH–H_2O–OHAc	[112]
	C18	MeCN–H_2O (coumarin derivatives)	[113,114]
	C18	MeCN–*n*-PrOH–H_2O–OHAc (32:4:63:0.1)	[115]
Soyasaponin VI	Ultrasphere C18	MeCN–OHAc (1000:0.3) (S1)	[116–118]
		H_2O–OHAc–EDTA (1000:0.3:0.15) (S2)	
Steroid saponin	Silica gel	Hexane–EtOH–H_2O	[119]

(Continued)

TABLE 25.1 (CONTINUED)
HPLC Determination of Saponins

Sample	Column	Solvent System	Ref.
	NH_2	MeCN–H_2O	[119]
Steridal sapog.	Silica gel	Hexane–Me_2CO, hexane–EtOH	[120,121]
	C18	MeCN–hexane–THF, MeOH–H2O	[120,121]
Steroidal sap.	Lichrospher C-18	CH_3CN–H_2O	[122]
	Lichrospher C-18	CH_3CN–H_2O	[123]
Swartzia sap.	C18	MeCN–H_2O–TFA (30:70 → 50:50)	[9]
Symplocososides	YMC-Pack ODS-A	MeOH–H_2O	[124]
		CH_3CN–H_2O	
Synallactosides	Silasorb C-18	EtOH– H_2O	[125]
Ternstroemiasides A–F	YMC ODS-H80	MeOH–H_2O	[126]
Tripterygium sap.	YMC-Park SIL-06	hexane–EtOAc	[127]
Triquetrosides	RP-18	MeOH–H_2O	[128]
Tropeosides A, B	RP-18	MeOH–H_2O	[128]
Triterpene sap.	Alltech C18	H_2O–MeCN (20–80%)	[129]
	Hydroxyapatite	MeCN–H_2O (87:13; 80:20)	[130]
Ursolic acid	Nova-Pak RP-18	MeOH–H_2O–OHAc	[131]
Zafaral	RP-18	CH_3CN–H_2O	[132]
Zanhic acid glc.	Spherisorb C18	H_2O–MeCN (10 → 90%) Br derivatives	[133]

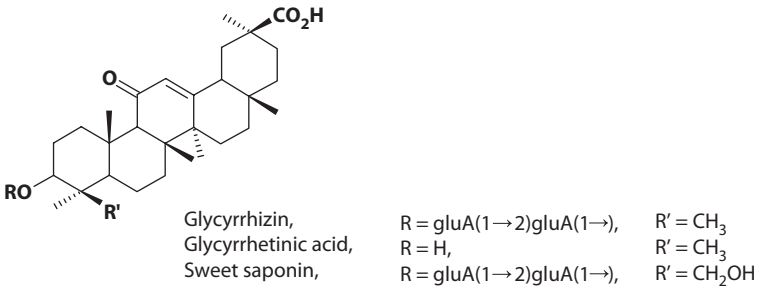

FIGURE 25.1 Chemical formula of glycyrrhetinic acid and its glycosides.

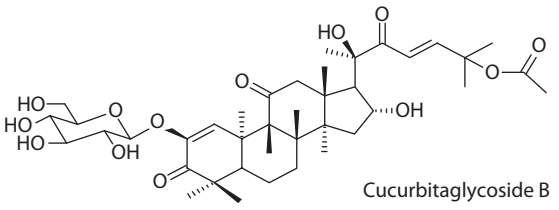

FIGURE 25.2 Chemical formula of cucurbitacine glycoside.

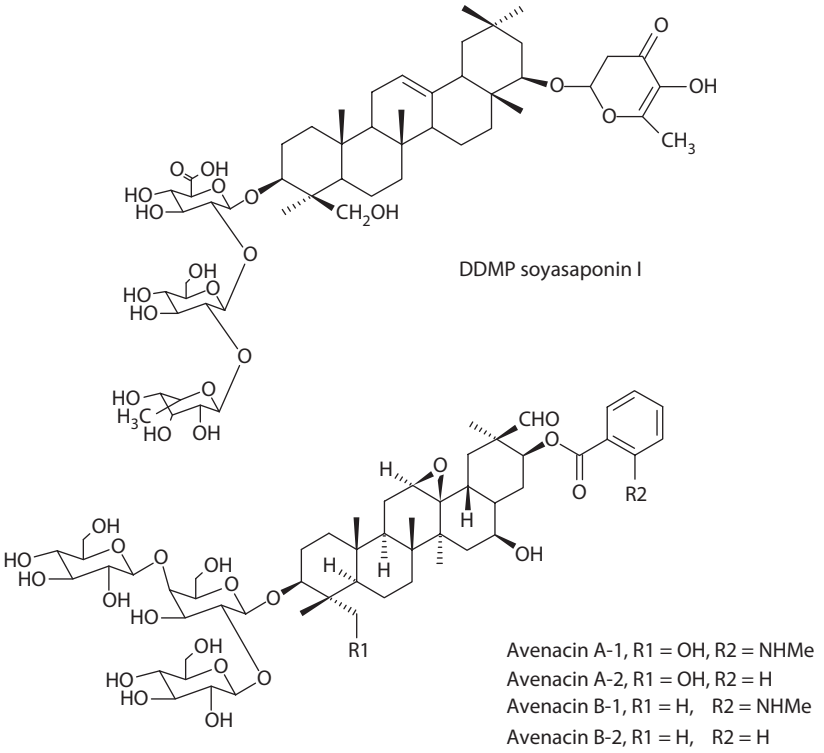

FIGURE 25.3 Chemical formula of 2,3-dihydro-2,5-dihydroxy-6-methyl-4-pyrone soyasaponin I (upper) and avenacins (lower).

overlap with the triterpenes, making determination difficult. The sensitivity of this detection mode has been satisfactory and, depending on the nature of the triterpene, ranges from 50 ng for avenacoside B [11] to 300 ng for ginseng saponins [37]. The detection at lower wavelengths, however, limits the selection of solvents and gradients that can be used. Since acetonitrile has much lower absorption at lower wavelengths than methanol does, acetonitrile–water gradients are the mode of choice. Similarly, the gradient cannot cover a wide range of concentrations due to the baseline drift, which creates an additional problem with triterpene analysis. The bidesmosidic saponins elute in a water–acetonitrile gradient at relatively low concentrations of acetonitrile, while monodesmosides elute later and are consequently much more difficult to quantify. Some plant extracts may contain large numbers of glycosides differing in polarity due to the number of sugars attached. Analysis of such a complicated mixture would need a wide range of solvent concentrations. Application of a gradient completely excludes detection with a refractive index detector (RI), so this type of detection has rarely been used. However, some oleane saponins have been successfully analyzed with RI, using small gradient changes in the eluting solvent of either methanol–water or acetonitrile–water on a C8 column and 210 nm detection [91].

25.3.2 Derivatization

The alternative to low-wavelength UV or RI detection has been precolumn derivatization that introduces a chromophore to the triterpene molecule and facilitates UV detection at higher wavelengths. Several different attempts have been made to introduce such a chromophore based on the substitution of different functional groups present in the molecules.

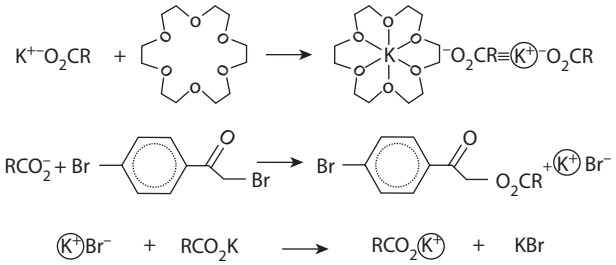

FIGURE 25.4 The scheme of the reaction for derivatization with bromophenacyl bromide.

25.3.2.1 Bromophenacyl Derivatization

One option was derivatization with bromophenacyl bromide in the presence of crown ether. The method was originally introduced for precolumn derivatization of fatty acids [136]. Thus, this can be applied exclusively to the triterpenes that possess a carboxylic functional group in the molecule. This group can be situated either on the aglycone or on a sugar substitution (uronic acids).

The reaction involved in derivatization is shown in Figure 25.4. The crown ether present in the solution has tremendous ability to complex metal salts, especially those of potassium, and to aid, by the solvation of the cation, the dissolution of salt in nonpolar, apriotic solvents, such as acetonitrile, benzene, cyclohexane, methylene chloride, and carbon tetrachloride. Anions of these salts have been shown to be unusually reactive, especially the carboxylate anions. The molar ratio of the alkylating reagent to crown ether is usually 20:1 or 10:1. The resulting derivatized compound can be obtained with a quantitative yield higher than 97% with no by-products. Rigorous anhydrous conditions are not necessary. The absorption maxima of derivatized compounds range between 257 and 261 nm, depending on the solvent, and their molar absorptivity at 254 nm range is between 17,000 and 19,000. The derivatized compounds are quite stable and, when stored at low temperature (–10°C), can be used for a period longer than one month.

The following protocol for preparation can be successfully used: 2.5 mg of triterpene was dissolved in 0.25 mL of methanol and 2 mL of water containing 1 mg of potassium. Hydrogencarbonate was added drop-wise with occasional sonication. The samples were lyophilized and treated with 1 mL of a mixture of 4-bromophenacyl bromide (3.5 g) and 18-crown-6 (680 mg) in acetonitrile (100 mL). This was then refluxed at 100°C for 90 min and purified on a silica gel Sep-pak cartridge by eluting with 10 mL dichloromethane followed by 10 mL chloroform–methanol (1:1). After evaporation of the latter solvent, the sample was redissolved in 2 mL of methanol and used for HPLC analysis. This protocol has been successfully used for the determination of oleane saponins in *Phytolacca dodecandra* [90] or medicagenic and zanhic acid as well as soyasapogenol B glycosides from alfalfa (*Medicago sativa*) [79, 133].

25.3.2.2 Benzoyl Derivatives

The benzoate esters have been developed as potentially useful derivatives in cases where a reactive hydroxyl group is present in the molecule of interest, for example, hydroxy steroids [137]. The method has also been used successfully for saponin analysis. The extinction coefficients for benzoates at an absorption maximum of 230 nm were usually greater then 10,000. The reaction was quantitative.

The following protocol for derivatization was developed: 5–50 mg of hydroxyl-containing steroid were dissolved in 4 mL of pyridine. A threefold molar excess of benzoyl chloride, based on the amount of hydroxyl group in the steroid to be derivatized, was added to the pyridine. The mixture was shaken thoroughly for 5 min and allowed to stand in a hot water bath at 80°C for 15 min, after which time the reaction was complete. The reaction mixture was then poured into a

reparatory funnel containing 50 mL of 0.1N HCl and 50 mL of ether. A triplicate wash with the acid removed the pyridine. A more dilute acid wash is recommended for steroids with Δ^5 configuration due to the possibility of forming cyclo-steroid artefacts. The ether phase is neutralized by distilled water, washed, and then extracted with two 50-mL portions of saturated sodium carbonate solution to remove excess benzoic acid. After a final water wash to remove excess carbonate, the ether phase containing the steroid benzoate is evaporated to dryness, dissolved in chloroform, and used for HPLC.

25.3.2.3 2,4-Dinitrophenylhydrazine (DNPH) Derivatives

DNPH derivatives were developed to enhance the sensitivity of carbonyl compounds to ultraviolet light. The reaction between DNPH and carbonyl groups is fast, selective, and quantitative. Using this method 17-keto steroids, including androsterone, epiandrosteron, etiocholanolone, and dehydroepiandrosteron, were successfully chromatographed [138].

25.3.2.4 Coumarin Derivatives

A method based on conversion of triterpene saponins to a coumarin derivative was employed for the separation of soybean saponins [113]. Derivatized compounds were chromatographed employing a C18 column, an acetonitrile–water gradient mixture, and fluorescence detection. The derivatization process requires a deesterification treatment of the saponin mixture prior to the chromatographic analysis. In the case of soyasaponins, where the glycosides are ether linked, this does not present a problem, but the technique cannot be used for saponins with ester-linked sugars.

25.3.3 Evaporative Light Scattering Detection

To overcome the problems of UV detection and develop validated analytical methods for quality control of some products, the evaporative light scattering detector (ELSD) can be used for detection of saponins. This detector was successfully applied for measuring soyasapogenol A and B in soybeans, which were separated on a C18 column with the mixture acetonitrile–propyl alcohol–water–acetic acid (CH_3CN-PrOH-H_2O-OHAc, 80:6:13.9:0.1) [105]. A validated HPLC method with ELSD detection was also developed for determination of the major ginsenosides in samples of Chinese traditional medicines [109]. Saponins were successfully separated on a Spherisorb ODS2, C18 column in an acetonitrile–water gradient and quantified using calibration curves, with detection limits of 50 ng.

A similar validated method using ELSD was developed for saponin determination in *Flos Lonicerae* (dried buds of several species of the genus *Lonicera*) used as an herb in traditional Chinese medicine [29]. Seven saponins (the macranthoidins A and B, macranthosides A and B, dipsacoside B, and two hederagenin glycosides) were successfully separated on a C18 column using an acetonitrile–water–acetic acid gradient and quantified.

25.4 LIQUID CHROMATOGRAPHY WITH MASS SPECTROMETRY

Difficulties in detection of saponins by methods using liquid chromatography (LC) with UV encouraged development of hyphenated techniques combining LC and mass spectrometry (LC-MS). Three different interfaces are used most frequently in LC-MS mode. These include thermospray (LC-TSP-MS), continuous flow fast atom bombardment (LC-CF-FAB) and electrospray (LC-ESI-MS) techniques. The most suitable one for triterpene analysis is ESI, since this allows analysis of molecules up to 2000 mu, which is the case when saponins are studied. Applying MS to structural and analytical problems has become increasingly common over the last few years, and MS has been extensively used to characterize, confirm, and determine saponins in plant extracts.

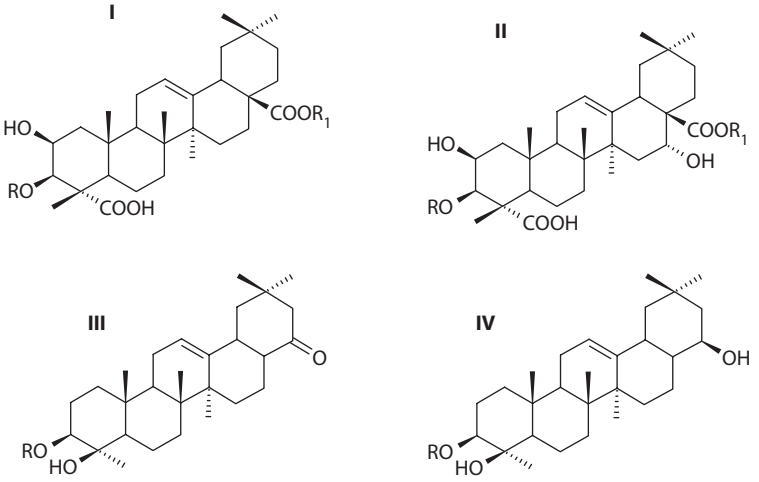

FIGURE 25.5 Chemical formulas of identified compounds: I, medicagenic acid; II, zanhic acid; III, soyasapogenol E; and IV, soyasapogenol B.

For the structural confirmation of saponins, ion trap MS^n (multistage mass spectrometry) is the solution of choice. The most extensive work using LC-MS was performed on commercially important plants, for example, soyasaponins in soybean products (*Glycine max*) [106,107,108] and black beans (*Vigna mungo* L. *Hepper*) [139], ginseng (*Panax notoginseng*) saponins [93,140], and recently *Medicago* saponins [81,82,141] because of their importance to the metabolomics and functional genomics of Fabaceae.

Two classes of saponins, namely, A and B, can be found in soybeans. Class A saponins possess soyasapogenol A in an aglycone part and have a bidesmosidic character (3 and 22-O substituted with up to four sugars). They also contain three or four acetyl groups in their genuine form. The saponins of class B are monodesmosidic structures possessing soyasapogenol B or E in an aglycone part. Likewise, the soyasaponins belonging to group B occur as both DDMP and non-DDMP forms. Both acetyl groups in type A and DDMP in type B saponins are very labile substitutions and can be partially lost during sample preparation, which additionally increases the number of saponins (artifacts) in a matrix. This diversity hinders the separation and determination of individual compounds; in fact, no method is available that can determine all genuine soybean saponins. To reduce complexity and increase stability of soybean saponins, partial alkaline degradation cleaving acetyl and DDMP groups prior to LC-MS analysis was proposed [106]. Such deacetylated and non-DDMP saponins could be easily quantified using selective ions of their $[M\text{-}H]^-$ ions. This procedure was improved, allowing fingerprinting of soybean saponins based on monitoring of the soyasaponin-specific protonated aglycones and dehydrated aglycone ions [107]. For type A saponins, these ions of soyasapogenol A (*m/z* 475) corresponded to peaks at *m/z* 457 $[A\text{-}H_2O]^+$, 439 $[A\text{-}2H_2O]^+$, and 421 $[A\text{-}3H_2O]^+$ and, for soyasapogenol B (*m/z* 457), corresponded to peaks 441 $[B\text{-}H_2O]^+$, 423 $[B\text{-}2H_2O]^+$, and 405 $[B\text{-}3H_2O]^+$. A characteristic continuously recorded positive ion in the total ion chromatogram (*m/z* 200–1500) allowed identification of several type A and B saponins and some isoflavones in one run and a quantification of the soyasaponin Bb based on the available Bb standard.

A combination of ELSD and ESI-MS was applied to develop a procedure for separation and determination of the group A soyasaponins with different degrees of acetylation and of the group B soyasaponins in both their DDMP-conjugated and nonconjugated forms occurring in soybeans [108].

For ginseng saponins, the situation seem to be even more complex than for soybeans. Up til recently 64 structures have been reported, and standards are available for only seven glycosides

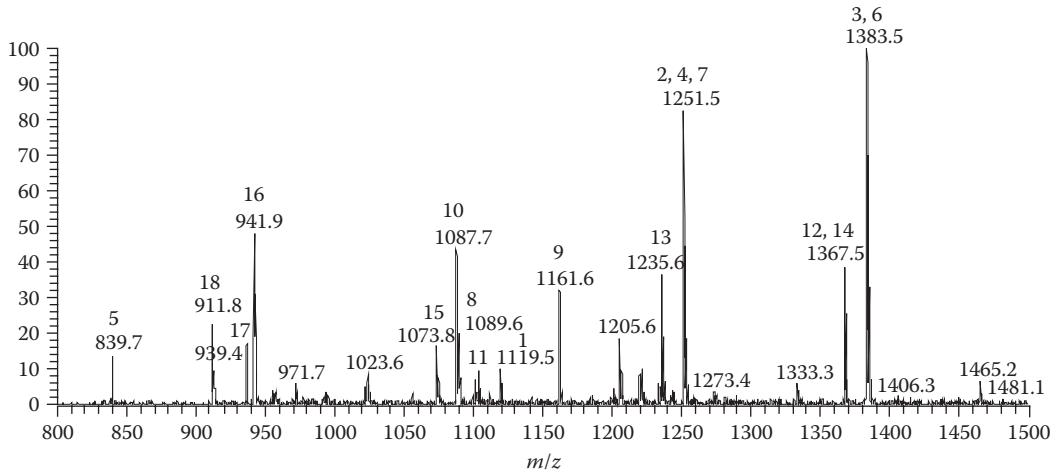

FIGURE 25.6 Direct-injection electrospray ionization mass spectrometry profile of the mixture of *M. truncatula* saponins purified by solid-phase extraction.

[140]. For such a complex mixture reliable methods for on-line characterization are essential. As shown recently, ginsenosides under ESI-MS form stable adduct ions with small alkali and transition metals. Negative-ion and MS/MS experiments allowed for the determination of molecular mass, type of triterpene core, type of sugars (pentose and hexose), and type of attachment points of the sugars to the core. MS analyses of ginseng root extracts [93] showed that the main ions observed were the sodiated molecular ions $[M + Na]^+$ and to a lesser extent $[M + K]^+$ ions. MS/MS experiments showed that cleavage occurred predominantly at the glycosidic linkage at C-20.

Barrel medic (*Medicago truncatula*), a close relative of alfalfa (*Medicago sativa*), has gained much attention recently as a model crop for research on the genomics of Fabaceae. Extensive work on the possibility of metabolite profiling using LC-ESI-MS/MS was performed on this plant. Comparison of the saponin profiles in barrel medic and alfalfa showed that the roots of barrel medic are a richer source of saponins than the roots of alfalfa [142]. In both species 30 individual compounds being glycosides of four major aglycones were identified (Figure 25.5) based on observed LC-ESI-MS profiles and LC-ESI-MS/MS experiments. Some saponins were quantified in roots and aerial parts of barrel medic based on the same techniques [82]. These trials, however, clearly show that for successful quantification of saponins with LC-ESI-MS, a set of original standards is essential. Lack of standards and dependence on fragmentation patterns may sometimes lead to misleading results.

In addition, analysis of fragmentation patterns does not provide information on the type of sugar and the place of its substitution. This only allows pentoses, hexoses, and uronic acids to be distinguished but not recognize different sugars in the same class. In LC-MS analysis the straight *off-line* injection of solid-phase-extracted saponins may be of great help in following interpretation of peaks in the total ion current (TIC) LC profile (Figure 25.6).

As shown for the *M. truncatula* saponin mixture, the 13 major peaks corresponding to $[M-H]^-$ ions of dominant saponins can be distinguished. Some peaks corresponded to two or three different compounds having identical *m/z* values, and these were recognized based on the retention times and selected ion chromatograms (SIC) in relation to original standards (Figure 25.7). Without appropriate standards this differentiation would not be possible.

The discussed examples of using LC-ESI-MS/MS for identification/confirmation and determination of saponins show that this technique is still a challenge and has several limitations. For the development of a reliable routine method, the availability of appropriate standards obtained by classical separation methods is essential. Even structural confirmation is not absolutely certain by this

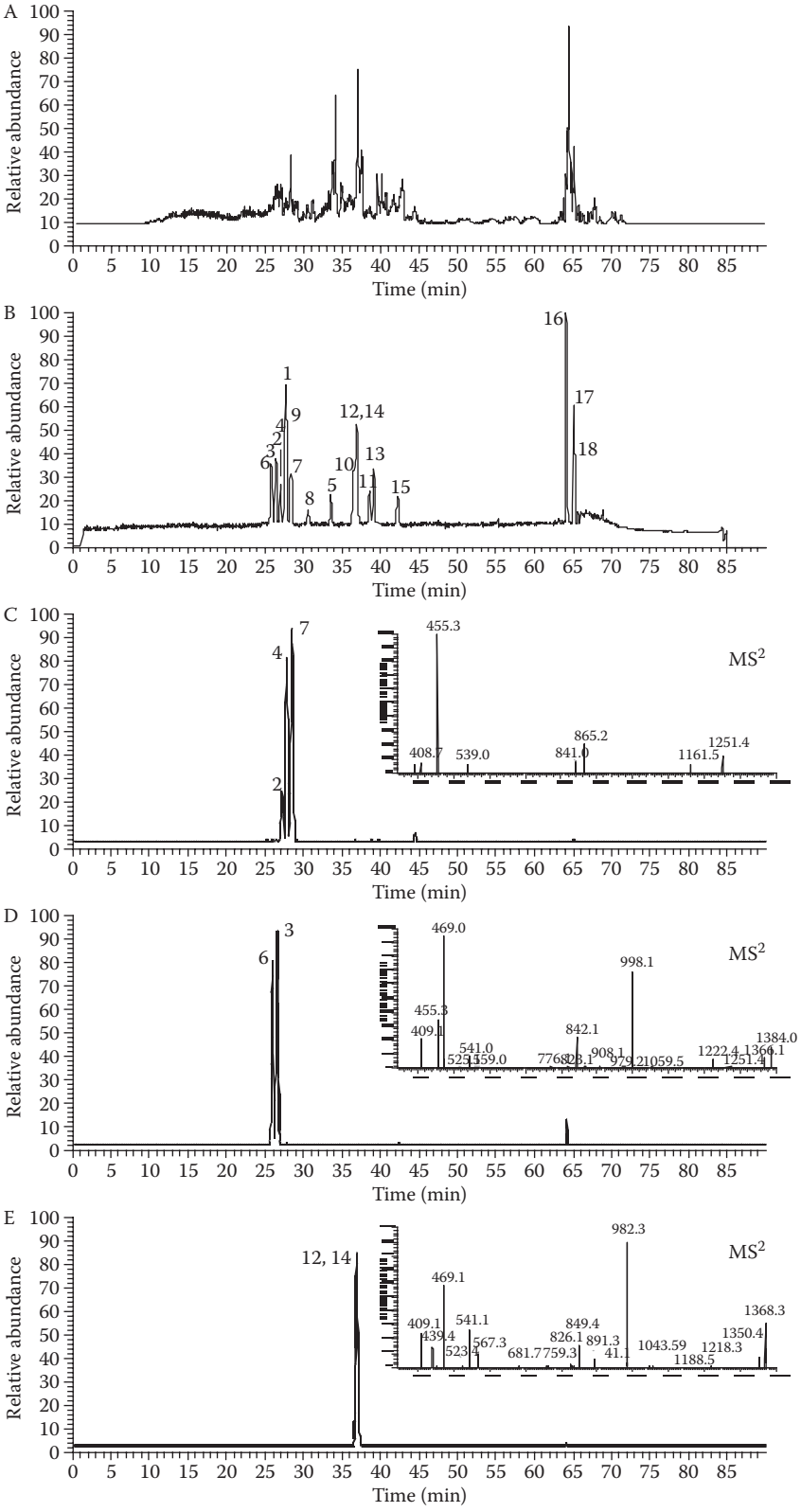
A
B
C
D
E
Relative abundance
Time (min)
MS2
455.3
865.2
408.7
539.0
841.0
1161.5
1251.4
469.0
998.1
455.3
409.1
842.1
541.0
1384.0
1366.1
982.3
469.1
409.1
541.1
849.4
826.1
891.3
567.3
1043.59
1218.3
1368.3
1350.4
1188.5

FIGURE 25.7 (Opposite) Total and selected ion chromatograms of *M. truncatula*, cultivar Jemalong A, aerial part saponins: (A) total ion chromatogram obtained by negative-ion liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) of plant extract purified by solid-phase extraction; (B) total ion chromatogram obtained by negative-ion LC-ESI-MS of a mixture of 18 standard saponins; (C) selective ion chromatogram (*m/z* 1257 $[M\text{-}H]^-$) representing saponins 2, 4, and 7; (D) selective ion chromatogram (*m/z* 1383 $[M\text{-}H]^-$) representing saponins 3 and 6; (E) selective ion chromatogram (*m/z* 1367 $[M\text{-}H]^-$) representing saponins 12 and 14. Peak areas from the selective ion chromatograms were used to calculate absolute concentrations based on the standard response curves of 18 saponin standards from aerial parts of *M. truncatula*. Tandem mass spectrometry (MS/MS) spectra were obtained by direct-introduction ESI-MS/MS.

technique. As shown in the research on barrel medic, identical LC-ESI-MS/MS sequencing of two compounds and identical retention times cannot distinguish between (1→2) and (1→3) sugar linkage, which differentiates some saponins of barrel medic and alfalfa.

25.5 CONCLUSION

The LC method seems to be most often acceptable for analysis of triterpenes. The lack of chromophores makes application of this method rather difficult. Derivatization has been very limited due to possible artifacts and structural requirements, so that nonspecific detection at 200–210 nm or ELSD have been the preferred techniques. However, they can be routinely used only in cases where appropriate standards are available and samples are purified of the other matrix components prior to analysis. The most reliable technique, giving the possibility of both quantitation and identification, is LC-MS/MS. But even in this technique, solid separation work to obtain purified standards is essential for reliable conclusions. Structural information from MS/MS analyses is quite limited; the type of sugar linkage and place of substitution cannot be determined. Moreover, the separation of compounds with the same number of sugars attached, but differing in the type of sugars, is difficult or even impossible. For quantitation, mass spectrometers with an ion trap detector are not recommended due to high signal instability. MS systems with quadruple detectors are preferable for this purpose. Furthermore, the development of new stationary phases for triterpenoid separation would be very helpful.

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26 HPLC of Carotenoids

Łukasz Cieśla

CONTENTS

26.1 DEFINITION, CHEMICAL CLASSIFICATION, OCCURRENCE, AND BIOSYNTHESIS OF CAROTENOIDS

Carotenoids are an important class of naturally occurring compounds that are widely distributed in nature. They are a class of hydrocarbons (carotenes, e.g., β-carotene, lycopene) and their oxygenated derivatives (xanthophylls, e.g., lutein, β-cryptoxanthin) consisting of eight isoprenoid units joined in such a manner that the arrangement of isoprenoid units is reversed at the center of the molecule so that the two central methyl groups are in a 1,6-positional relationship and the remaining nonterminal methyl groups are in a 1,5-positional relationship [1]. All carotenoids may be formally derived from the acyclic $C_{40}H_{56}$ structure having a long central chain of conjugated double bonds by (i) hydrogenation, (ii) dehydrogenation, (iii) cyclization, or (iv) oxidation, or any combination of these processes. Carotene comes in two primary forms designated by characters from the Greek alphabet: alpha-carotene (α-carotene) and beta-carotene (β-carotene). Gamma, delta, epsilon, and zeta (γ-, δ-, ε-, and ζ-carotene) exist as well. The rules for the semisystematic nomenclature can be found elsewhere [1,2]. As hydrocarbons that contain no oxygen, carotenes are fat-soluble but insoluble in water (in contrast with other carotenoids, such as xanthophylls, which are slightly less chemically hydrophobic).

Carotenoids are widespread among the higher plants; however, some bacteria, algae, and fungi can also synthesize these fat-soluble pigments. Although animals appear to be incapable of synthesizing

carotenoids, many animals incorporate carotenoids from their diet. Within animals, they provide bright coloration, serve as antioxidants, and can be a source for vitamin A activity.

26.2 BIOLOGICAL AND MEDICAL SIGNIFICANCE

In photosynthetic organisms, specifically flora, carotenoids play a vital role in the photosynthetic reaction center. They either participate in the energy-transfer process or protect the reaction center from auto-oxidation. Humans are not able to synthesize these constituents and must obtain them through their diet.

Many potential beneficial health properties of carotenoids have been described in the literature. The most important, from the medicinal point of view, is that some carotenoids are active as provitamin A, which is essential for good eyesight. Provitamin A carotenoids (pVACs) are subsequently converted to the active forms of vitamin A by the activity of the intestinal mono-oxygenase. Only a few of the known carotenoids have the β-ring end-group structure necessary for vitamin A activity. The most important pVACs are all-*trans* α-carotene, all-*trans* β-carotene, and β-cryptoxanthin [3]. Vitamin A is essential for normal development of children, and deficiency can lead to night blindness as well as resulting in an increased susceptibility to a variety of other diseases due to a weakened immune system [3]. Furthermore, carotenoids are very potent free-radical scavengers, and thus they are able to protect humans from cardiovascular diseases, cancer, or macular degeneration [4]. These substances have also been applied with success in treating patients suffering from erythropoietic protoporphyria [5,6], as well as being proved to stimulate the immune response at different levels, inhibit tumor cells growth, among other functions.

26.3 EXTRACTION METHODS AND SAMPLE PRETREATMENT

The major source of error in carotenoid analysis is reportedly due to differences in sample-preparation methods [7]. Carotenoids can be found in plant tissues in their free form or in a more stable fatty acid esterified form. Thus in carotenoid analysis, it should be taken into account that the extract may contain free carotenoids as well as their different esters with different fatty acids.

Another problem in the analysis of these compounds is their instability, caused by the presence of the conjugated double bond system [8]. They are especially susceptible to degradation from light, heat, oxygen, and acids. All the steps of the extraction and sample pretreatment should be validated to check whether the analyzed carotenoids undergo chemical changes. Several precautions are necessary when handling carotenoids, too. To prevent oxidation, it is common to use antioxidants with the extraction solvents, for example, pyrogallol [9,10], ethoxyquin, ascorbic acid, sodium ascorbate, butylated hydroxyanisole [10], or, most frequently, BHT (butylated hydroxytoluene). The last of the reagents is normally used at concentration from 0,01 to 0,1% in the extraction solution. All the extraction steps should be carried out in dim light in order to avoid contact with sunlight. Evaporation should be performed under reduced pressure, optimally under a stream of nitrogen at a temperature not higher than 40°C [4]. All the samples should be kept in the dark, at a temperature of about –20°C [8].

26.3.1 Extraction

Sample pretreatment begins with the extraction of a sample of carotenoids, dependent both on the tissue matrix and on the carotenoid species of interest [7]. Unfortunately, there is no standard procedure for the extraction of carotenoids due to the wide variety of sample types and composition [11]. Usually methanol (MeOH) or a mixture of it with less polar solvents is used as the extraction solvent. The following mixtures have been reported: MeOH + tetrahydrofuran (THF) [12,13], MeOH + diethyl ether, MeOH + chloroform ($CHCl_3$) [14], MeOH + hexane, and MeOH + acetone + hexane [8]. There are also reports on the use of acetone alone [15,16] or in combination with

light petroleum [17,18], THF [19], ethanol (EtOH) [20], or hexane [21], as well as more complex solvent mixtures, such as hexane + acetone + MeOH + toluene (10:7:6:7, v/v). Mendez-Robles et al. used hexane for complete extraction of carotenoid pigments from seeds [22]. Fraser et al. reported that chloroform was the best extraction solvent for pigment isolation from plant leaf tissue [23]. It was found to provide 10-fold higher carotenoid extraction capability from the plant tissue. Several disadvantages of the mentioned solvents should be taken into account before one is chosen to be used in the extraction step. For example, acetone is highly volatile, and so concentration effects can be observed, due to evaporation [7]. THF has been reported to produce peroxides on standing that could lead to carotenoid breakdown [7]. Davey et al. recommends using hot (85°C) EtOH plus BHT for tissues containing small amounts of carotenoids and THF:MeOH (1:1, v/v) plus BHT at room temperature for carotenoid-rich samples [7]. There is a report stating that acetone may cause carotenoid degradation [24].

Davey et al. investigated the influence of different extraction solvents on the yield of carotenoids [7]. The authors reported there were no significant differences in the yields obtained for any of the solvents, in contrast to the work of van Jaarsveld et al., who reported that THF:MeOH (1:1, v/v) was the most efficient extraction solvent [25]. Kimura et al. state that extraction with THF:MeOH (1:1) gave significantly lower results for zeaxanthin and lutein compared to extraction with acetone [26]. The authors conclude that this was not due to the extraction efficiency but to a problem in the partition to petroleum ether, as the dihydrocarotenoids are more difficult to transfer from THF:MeOH to an apolar solvent than from acetone.

Carotenoids are usually extracted from the sample by homogenization with the proper solvent [13]. In the next step the carotenoids are extracted from the MeOH:THF mixture by the use of petroleum ether containing BHT [13]. Increased extraction hydrophobicity limits the amount of extract that can be directly injected onto the high performance liquid chromatography (HPLC) system, as with the more hydrophobic injection solvents, the carotenoids are less well retained, which leads to peak broadening and/or peak splitting. More detailed information on the influence of solvent type on the maximum injection volume can be found in ref. [7].

Some researchers reported the addition of solid magnesium carbonate into the extraction liquid to neutralize any organic acids [12,13,27]. Kurz et al. described a method of carotenoid extraction from samples containing high amounts of sugar [10]. For their removal the sample should first be extracted with MeOH, then the carotenoids are extracted with hexane, while the sugars remain in the MeOH layer. Weber et al. recommend using dimethylsulfoxide (DMSO) sample incubation followed by pigment extraction from yeast [28].

Supercritical fluid extraction has recently gained more attention in the extraction process of carotenoid-containing plants. Usually CO_2 with 1% of a polar modifier, such as MeOH, EtOH, or 2-propanol, was applied. In all cases better recoveries were reported compared to traditional liquid extraction. Machmudah et al. investigated different factors influencing the supercritical fluid extraction of carotenoids from *Rosa canina* [29]. The amount of extracted carotenoids increased with increasing pressure, temperature, and CO_2 flow rate, with several exceptions: For example, increasing the CO_2 flow rate resulted in decreasing β-carotene extraction [29]. The application of statistical analysis of variance (ANOVA) showed temperature was the most influential variable on the yield of total carotenoids. The optimum conditions for lycopene extraction were 80°C, 450 bar, and 3 mL/min and, for β-carotene and lutein, 60°C, 450 bar, and 3 mL/min. The potential benefits of supercritical fluid extraction for lycopene from tomato samples have been described in several papers [30–34].

After the extraction step, samples usually contain polar contaminants that can be removed during saponification or by partitioning the extract into water or aqueous salt solutions. To assess losses during the extraction procedures, an internal standard is usually added to the analyzed sample. For example, β-apo-8'-carotenal can be used; however, it may not be applied in the case of green plant parts since it coelutes with chlorophyll B. In that case echinenone can be a good alternative [13].

26.3.2 Saponification

In carotenoid analysis, saponification is commonly done prior to the analysis to transform all the components into their free form [4]. Usually, KOH aqueous, methanolic, or ethanolic solution is applied at concentrations from 10 to 60% (w/v). It is advised to conduct this process under nitrogen in the dark [13]. This procedure also allows the analysis of the carotenoid fraction, eliminating analytical interferences from other compounds. When the identification of all the peaks in the chromatogram is not needed, and the chromatographic system separates the chlorophylls from the carotenoids, saponification is not necessary [13].

Saponification may lead to losses, isomerization, or other reactions. It has been observed that saponification losses were the highest (≈25%) for lycopene [9]. Oliver et al. confirmed that this process results in the underestimation of some carotenoids [35]. Khachik et al. investigated the effect of saponification on both qualitative and quantitative distribution of carotenoids in green vegetables by evaluating HPLC profiles before and after alkali treatment (see Figure 26.1) [36]. The quantitative evaluation showed that saponification was accompanied by significant loss of xanthophylls, particularly the epoxycarotenoids, while losses of carotenes were not significant. Kimura et al. observed that even mild saponification may lead to significant degradation of lutein, zeaxanthin, and violaxanthin [37]. However, saponification of carotenoid extracts hydrolyzes all carotenoid esters and allows the detection of some simple carotenoids and other minority nonidentified peaks that were not detected in the nonsaponified extract [35]. Another disadvantage is that it involves extra handling steps, including the partitioning out of the saponified carotenoid species, drying down, and resolubilization of extracts before injection onto the HPLC [7].

Saponification is normally carried out at ambient temperature and is quite lengthy, as the sample has to be left under stirring overnight. To reduce the analysis time, the sample can be heated; however, there is a risk of artifact formation. Carotenes have been found to be more resistant against alkali than xanthophylls [9]. The saponification is followed by extraction of carotenoids with diethyl ether or hexane, and the extract is washed with water until the pH is neutral. De Sa and Rodriguez-Amaya report that lutein is lost during the washing that follows the saponification, and they propose several steps to avoid this loss [38]. The components of less complex samples can be handled without saponification, as the gradient elution mode employed is usually sufficient to remove the lipids from the column [26]. Sample defatting by precipitation has also been proposed, in which case the saponification step can be omitted [28]. In the case of analysis of carotenoid esters, this sample-pretreatment step is omitted, which substantially reduces the analysis time [4].

26.3.3 Standard Preparation

There are few commercially available carotenoid standards, and their purity is sometimes insufficient for chromatographic analysis. Thus, there is sometimes a need to prepare "self-made" standards. Barua described several methods for extracting the most commonly encountered carotenoids and their esters from different plant materials [15]. Lutein esters were extracted with acetone from marigold flowers. Lutein can be prepared by saponification of lutein esters or by treating the dry petals of marigold flowers with methanolic NaOH and further extraction with hexane. Zeaxanthin esters were obtained from Gou Qi Zi berries through repeated extraction with the following solvents: water, acetone, and hexane. Zeaxanthin was prepared in the same way as described for lutein. For lutein 5,6-epoxide preparation, lutein was dissolved in diethyl ether and stirred at room temperature with 3-chloroperoxybenzoic acid (for details see ref. [15]). The identity of lutein 5,6-epoxide was confirmed by treatment with a trace of dilute HCl, which converts the 5,6-epoxide quantitatively to the 5,8-furanoid compound; as a result, a hypsochromic shift was observed. Violaxanthin can be obtained through the reaction of zeaxanthin with 3-chloroperoxybenzoic acid followed by crystallization. Chen et al. obtained neoxanthin and violaxantin from spinach using preparative thin-layer chromatography (TLC) [39]. The authors also describe a method for obtaining isomeric forms of lutein and β-carotene, to be used as standards.

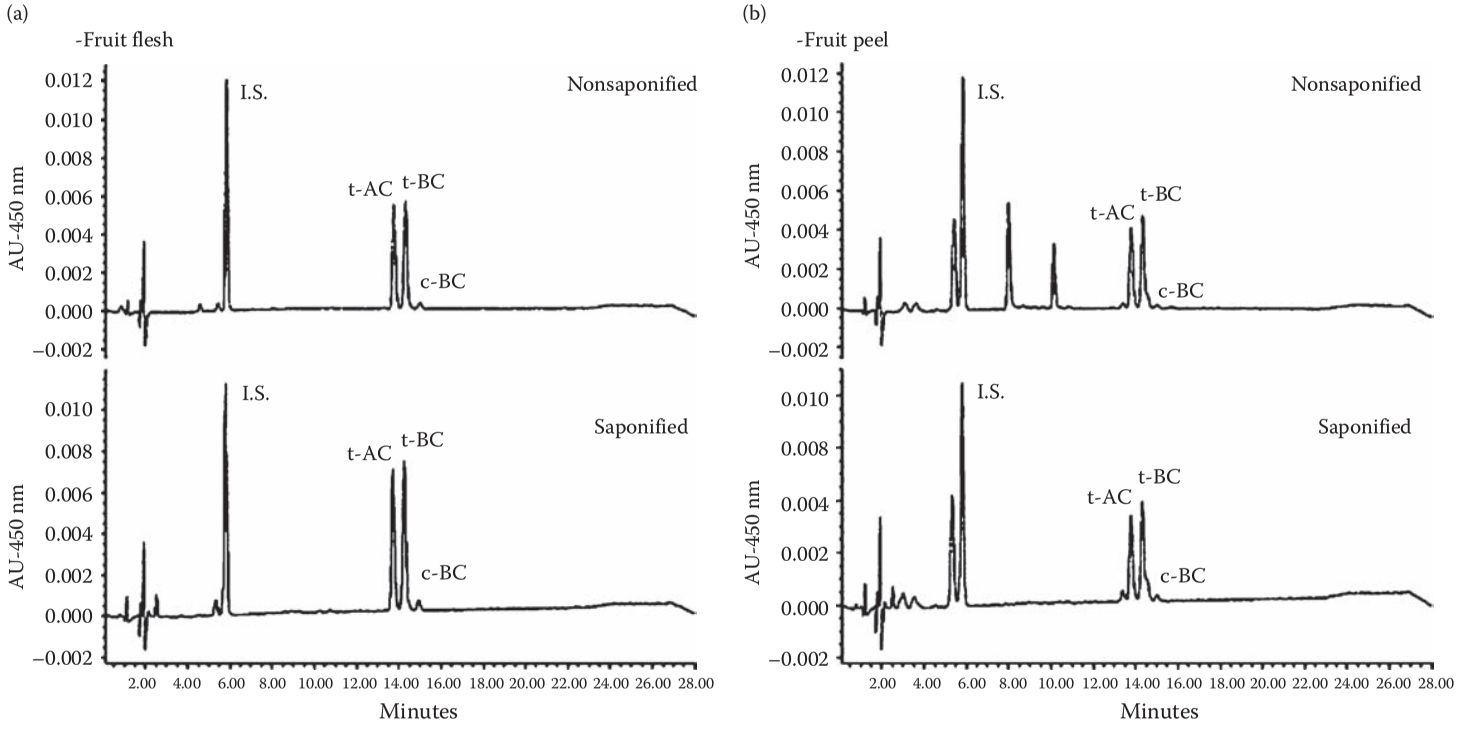

FIGURE 26.1 Effect of saponification on the reversed-phase HPLC profiles of (A) Musa pulp carotenoids and (B) peel carotenoids. Aliquots of tetrahydrofuran:methanol (1:1, v/v) extracts from lyophilized Mbouroukou-1. Peaks were identified according to their characteristic absorption spectrum and retention times. t-AC, all-*trans* α-carotene; t-BC, all-*trans* β-carotene; c-BC, *cis* β-carotene. (From Davey, M.W., Keulemans, J., and Swennen, R., *J. Chromatogr. A*, 1136, 176–184, 2006. With permission.)

Kimura and Rodriguez-Amaya described a scheme for obtaining standards and HPLC quantification of carotenoids [16]. The authors used an open-column system to isolate carotenoids from leafy vegetables. For example, lactucaxanthin (ε,ε-carotene-3,3'-diol), a carotenoid found only in lettuce, was obtained from that vegetable. If lactucaxanthin is not to be used in the analysis, the authors recommend cress or parsley, vegetables with a high carotenoid content, for obtaining of standards. For greater ease of isolation and higher amounts of standards, carotenoid-rich foods can be used as sources of standards, such as α-carotene and β-carotene from carrots, β-cryptoxanthin from papaya, and lycopene from tomato [16]. To obtain standards, carotenoids were extracted with cold acetone, partitioned to petroleum ether, concentrated in a rotary evaporator, and separated in an open column of MgO; for details see ref. [16]. Lutein and violaxanthin are the most difficult to separate; if pure standards cannot be obtained on magnesium oxide (MgO), the fraction corresponding to these carotenoids should be rechromatographed on a neutral alumina column using 25–40% acetone in petroleum ether to elute lutein and acetone to elute violaxanthin. The use of an open-column packed with MgO:Celite (1:1, activated at 120°C for 2 h) to obtain carotenoid standards has also been reported [40].

Hakala and Heinonen used tomato puree to isolate lycopene [41]. Solid-phase extraction, on silica cartridges, was followed by three purifications with semipreparative HPLC. However, the purity of the obtained standard was rather poor, only 70%. Breithaupt described a method of preparing selected carotenoid standards using semipreparative HPLC on a C_{30} column with methyl *tert*-butyl ether (MTBE):MeOH in the ratio 30:70 (v/v) for lutein and capsanthin and 70:30 (v/v) for lycopene [42]. Oliver et al. proposed a method for obtaining capsanthin and capsorubin standards from the extract of *Capsicum annum* fruit [35].

The preparation of a zeinoxanthin standard was described by Hamano and Mercadante [43]. After the extraction and saponification of frozen pulp of caja, the extract was applied to an open-column packed with neutral alumina, then one of the fractions, containing monohydroxy carotenoids, was submitted to TLC on MgO/kieselguhr (1:1). The isolation of β-carotene from sweet potato and of standards of β-cryptoxanthin, lutein, and zeaxanthin from green maize, by column chromatography, has been described by Kimura et al. [26].

Many flowers are also good sources of carotenoids [44]. For example, eschscholtzxanthin can be isolated from Californian poppy (*Eschscholtzia californica* Cham.), neoxanthin from laburnum (*Laburnum* sp.), lutein from marigold (*Tagetes erecta* L.), and so on [45,46]. Melendez-Martinez et al. describe a method of obtaining a whole set of different carotenoids from spinach and red pepper [47]. They describe a technique to convert violaxanthin into luteoxanthin, as well as a method of obtaining auroxanthin.

Identification of the obtained carotenoid standards can be performed using several methods, for example, on the basis of the retention times, co-chromatography with authentic samples, and absorption spectra. Xanthophylls' identification can be confirmed by chemical tests, such as acetylation of secondary hydroxyl groups with acetic anhydride and methylation of allylic secondary hydroxyl groups with acidic methanol. The progress of these reactions can be monitored on a silica gel thin layer or ascertained spectrophotometrically [48]. To help identify the peaks in the chromatograms, plant extracts can be analyzed before and after treatment with hydrochloric acid and sodium borohydride to check the presence of 5,6-epoxy carotenoids and ketocarotenoids or carotenols [47]. The minimum criteria for the identification of carotenoids can be found in the work of Schiedt and Liaaen-Jensen [49]. Azevedo-Meleiro and Rodriguez-Amaya describe several techniques applied to confirm the identity of the carotenoids in fruit samples [48].

26.4 HPLC SYSTEMS USED IN CAROTENOID ANALYSIS

Reversed-phase HPLC (RP-HPLC) has been the method of choice for carotenoid analysis, with the use of both C_{18} and C_{30} stationary phases. There are also reports on the use of C_8 columns for the resolution of different carotenoid classes [50]. Selectivity in carotenoid separation is influenced mainly

by stationary-phase bonding density, its chemistry (monomeric vs. polymeric surface modification), and alkyl chain length [51]. In addition, free silanols influence the separation of polar carotenoids, while nonpolar carotenoids are relatively insensitive to this stationary-phase property. Polymeric and more densely bonded stationary phases have been proved to provide better separations of carotenoid geometric isomers [51].

In reversed-phase liquid chromatography (RPLC), carotenoids are eluted according to their increasing hydrophobicity and decreasing polarity [4]. The elution order also depends on the type of cyclization present in the compound. On reversed-phase columns, polar carotenoids with two hydroxyl groups, such as lutein, elute earlier, followed by monohydroxy carotenoids (e.g., zeinoxanthin and β-cryptoxanrhin) and finally by the carotenes [43]. Carotenoids with 10 conjugated double bonds (c.d.b.), such as zeinoxanthin and α-carotene, are eluted earlier than those with corresponding polarity and 11 c.d.b, e.g., β-carotene and β-cryptoxanthin. According to several researchers the elution order of the β-carotene *cis* isomers can change depending on the characteristics of the C_{18} column and mobile phase employed [43,52–55].

Photodiode array detection is recommended in the case of carotenoids, as the identification of the pigments is easier due to rapid and advanced spectra analysis [35]. This detection method is able to quantify each peak at its own maximum absorption. Hamano and Mercadante emphasize the importance of a photodiode array detector for samples containing colorless carotenoids, such as phytoene, to avoid misleading identification [43]. However, during the analysis of carotenoid samples, one should always remember that some carotenoids with a different number of carbon atoms or a different functional group show the same absorbance spectrum [56]. For example, β-carotene and zeaxantin, which have the same chromophore but different hydroxyl groups, show an equivalent absorption spectrum. However, the maxima of the compounds' spectra are defined by the presence of particular chromophores, and the chromatographic behavior may be determined by differences in the carbon number or the presence of different functional groups between them [22]. Identical or comparable chromophores may lead to inconclusive or even erroneous identification, as Pfander et al. report [57]. UV detection at 450 nm is recommended by the majority of authors to determine the presence of carotenoids in the analyzed samples [12].

26.4.1 RP-HPLC on C_{18} Columns

C_{18} columns have been used in both isocratic and gradient (linear and step) elution modes. Due to carotenoids' apolarity, the application of nonaqueous eluents in RP-HPLC is a common approach. In the case of isocratic separations, eluents consisting of MeOH and less polar solvents have been commonly applied, for example, mixtures of MeOH with acetone [9], acetonitrile (MeCN) [9,12], or ethyl acetate (AcOEt) [12]. Hamano and Mercadante carried out the separation on a C_{18} column with 100% MeOH as the mobile phase [43]. Nyambaka and Ryley used a tertiary mixture comprised of MEOH, dichloromethane, and water to resolve carotenoids from different vegetables on a C_{18} column [52]. Some separations were performed with MeCN instead of MeOH [29]. In the gradient elution mode, eluents consisting of MeOH or MeCN mixed with less polar solvents were most commonly applied [9,17]. De Sa and Rodriguez-Amaya recommend a concave gradient for tissues with a complex carotenoid composition [38]. Melendez-Martinez et al. indicate that an isocratic chromatographic method on C_{18} columns may lead to erroneous identification of some carotenoids, for example, neoxanthin [58]. Coelution of lutein and zeaxanthin is also observed in the previously mentioned systems. Weber et al. state that C_{18} columns should be used rather than C_{30} ones when the resolution between *cis*/*trans* geometric isomers is not strictly required or the occurrence of natural *cis* isomers is not expected [28].

It is important to note that some carotenoids' absorbance maxima change in different solvents [59,60]; for example, they are blue-shifted in the presence of dichloromethane [56]. Zang et al. report that lutein shows absorbance spectra that are almost independent of solvents at various concentrations, while zeaxanthin, lycopene, and β-carotene were found to be more solvent-dependent

[59]. The authors conclude that the absorbance changes result not only from differences in molar extinction coefficients in different solvents but also from carotenoids' different solubility in the investigated solvents. For example, zeaxanthin exists as a monomer in MeOH and forms small crystals in hexane. It is interesting that lutein, which differs from zeaxanthin by only the position of one double bond is well soluble in hexane [59].

The choice of the injection solvent for carotenoids also plays an important role in the analysis [61]. Khachik et al. showed that injection solvents such as methylene chloride, chloroform, THF, benzene, and toluene can result in distortion of the HPLC peaks of carotenoids [62]. The polarity and solubility properties of the injection solvent and the mobile phase should be compatible. In the case of lycopene, to avoid degradation, it should be dissolved just before the analysis [13].

Bononi et al. reported the use of an RP-Amide (C_{16}) column and MeOH as the mobile phase to analyze the carotenoid-containing extract of *Dunaliella salina* [63]. Resolution of α-, all-*trans*, 9-*cis*, 13-*cis*, and 15-*cis* β-carotene was obtained with the applied chromatographic system.

Some researchers recommend using guard columns to prevent column contamination. A C_{18} guard column preceding the analytical column has been used in the analysis of carotenoids [15]. Scott reported that metal surfaces, particularly stainless steel frits in guard and analytical columns, are damaging to carotenoids [64]. Metal-free columns and polyether ether ketone (PEEK) tubing for column connections have been recommended [13,65].

26.4.2 RP-HPLC on C_{30} Columns

The use of C_{30} stationary phases is becoming more and more popular due to their good separation power and resolution, particularly for the analysis of the less polar carotenoids. They are often a better alternative to normal-phase columns for the separation of isomers since they are not as sensitive to the water content of the mobile phase and are not as susceptible to column fouling. C_{30} columns show significantly greater shape selectivity compared to C_{18} phases due to their rigid, highly ordered C_{30} alkyl groups [66–68]. This makes them the ideal HPLC column for the separation of carotenoids. For best performance, C_{30} columns should be used at ambient or lower temperatures. At elevated temperatures, the C_{30} alkyl chains will become less ordered and lose their shape selectivity. In addition, C_{30} phases should be used with mobile phases containing at least 20% organic modifier to avoid phase collapse.

As already stated, carotenoid hydrocarbons are better resolved with polymeric C_{18} columns than monomeric ones. However, *cis/trans* β-carotene isomers remain unresolved with a polymeric C_{18} column. Baseline separation of almost all isomers was achieved after the application of the same mobile phase on a C_{30} column (see Figure 26.2) [51]. Among the hydrocarbon carotenoid standards, lycopene has the most significant change of its retention behavior depending on column type [51]. C_{30} columns are usually recommended for complex samples containing high concentrations of *cis* isomers [7]. For samples with a simple carotenoid profile, C_{30} columns seem to provide little advantage over the use of conventional C_{18} RP-HPLC columns [7]. Different chromatographic behavior of *cis* and *trans* isomers of carotenoids was observed after the application of MeOH or MeCN as modifiers. Xanthophyll retention is also influenced by the presence of water. Good results were obtained on a C_{30} column with a nonaqueous MeOH:MTBE gradient. Acetone has also been used in combination with MeOH to reduce lycopene retention on a C_{30} column [66]. C_{30} columns are normally used in gradient elution mode [10,42,69–71] similarly to the analyses of carotenoids performed on C_{18} columns [72].

Li et al. investigated the influence of different solvent ratios, used in HPLC linear gradient elution mode on a C_{30} column, on the separation of lutein and zeaxanthin, a pair of carotenoids that are difficult to resolve [69]. A mobile phase with 60% MeOH, 33% MTBE, and 7% water (with 1.5% ammonium acetate (NH_4Ac) in the water) was optimal for the separation of xanthophylls with unidentified components in different plants [69]. Bell et al. reported the use of C_{34} stationary phases for carotenoid analysis. The resolution of investigated compounds was slightly improved for the C_{34} column [73].

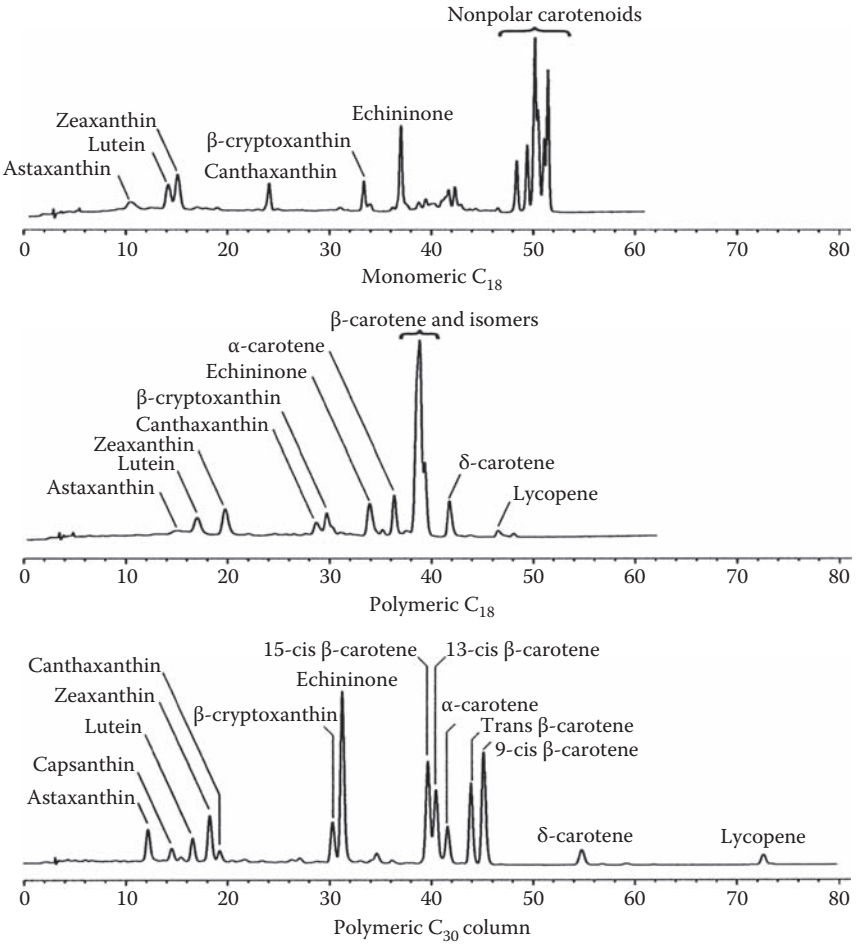

FIGURE 26.2 Separation of carotenoid standards on a monomeric C_{18} column, polymeric C_{18} column, and polymeric C_{30} column. For experimental data see ref. (From Sander, L.C., Sharpless K.E., and Pursch, M., *J. Chromatogr. A*, 880, 189–202, 2000. With permission.) Time scale in minutes.

26.4.3 Temperature Influence on Carotenoid Separation

Temperature is another factor influencing carotenoid separation. Several papers have described changes in column selectivity related to temperature in carotenoid analysis [66,74–76]. The separation behavior of C_{30} phases is strongly temperature-dependent, with the best separations being obtained at lower temperatures [66]. Albert presents an example of the influence of temperature on carotenoid resolution on C_{30} columns [66]. The baseline separation of all-*trans* and 9-*cis* β-carotenes is obtained at 295 K. An increased selectivity is obtained at 285 K, but the retention times are quite high. When temperature rises, the selectivity values decrease, leading to coelution of the investigated isomers at 315 K (see Figure 26.3). The author concludes that the strongly temperature-dependent separation characteristics of polymeric C_{30} bonded phases are due to the alkyl chain arrangement present at the silica surface [66]. Unusual changes in retention with a C_{30} column have been observed, for example, in the case of 13-*cis* and 15-*cis* α- and β-carotene, which are more strongly retained as the temperature rises. Zhu et al. reported that, with monomeric C_{18} columns, temperature changes influenced selectivity more than mobile-phase composition [76]. As the temperature has a significant influence on column selectivity, it should be controlled to improve reproducibility. Temperature control may also prove useful for selectivity tuning in method development [51].

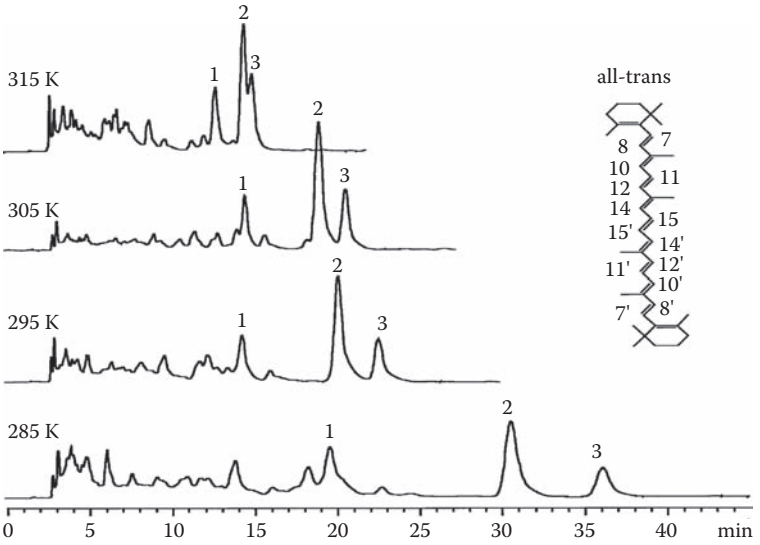

FIGURE 26.3 HPLC separations of L-carotene isomers at different temperatures with a C_{30} stationary phase: (1) 13-*cis*; (2) all-*trans*; (3) 9-*cis* L-carotene. Mobile phase: methanol/methyl *tert*-butyl ether (75:25, v/v), flow rate 1 mL/min; detector: UV, 450 nm. (From Albert, K., *Trends Anal. Chem*., 17, 648–658, 1998. With permission.)

26.4.4 Mobile-Phase Additives

Almost all carotenoids can decompose, undergoing isomerization or dehydration when subjected to acid conditions. Carotenoids containing a 5,6-epoxy-β ring can easily be converted into corresponding 5,8-epoxides in the presence of even traces of acid. Underivatized free-acidic silanols may catalyze these reactions. To prevent this, some authors recommend the addition of small amount of amines into the mobile phase, such as triethylamine (TEA) [13]. For example, the eluent MeCN:MeOH:AcOEt (70:10:9, v/v/v) containing 1% TEA, was applied in the isocratic separation of carotenoids from *Rosa canina* fruits [17]. This eluent was used along with MeOH–MeCN–hexane–dichloromethane (DCM) (50:12:10:8) containing 0.5% TEA in gradient separation [17]. The addition of TEA also causes a reduction in retention times without affecting the baseline separation of the main carotenoid peaks. Incorporation of amine into the mobile phase is also responsible for reducing free silanols' acidity, which may improve the results obtained in the case of polar xanthophylls. The addition of BHT into the mobile phase at a concentration of 0.1% is sometimes carried out to prevent carotenoids from oxidation during chromatography [12]. BHT has no discernible impact on peak resolutions/separations [7].

The use of ammonium acetate, usually at a concentration of 10 mM, as a mobile-phase additive has also been reported by several authors [15,77]. It is used to reduce the tailing of compounds with carboxyl functions [78]. The acidic character of bixin and norbixin means that special attention has to be given to their determination in RP-HPLC systems [42]. Ion-pair reagents have occasionally been incorporated into the mobile phase. Tetrabutyl ammonium hydrogen phosphate (TBAH) was added to the eluent (0.1%, w/v); however, a disadvantage of a system incorporating this reagent is a significantly reduced recovery of β-carotene and lycopene [42]. Cichelli and Pertesana report the utilization of tetrabutylammonium chloride as a mobile-phase additive at a concentration of 0.05 M [77].

26.4.5 Hyphenated Methods

For very complex samples, hyphenated methods enable the resolution and proper identification of sample components. As far as carotenoids are concerned, comprehensive two-dimensional liquid

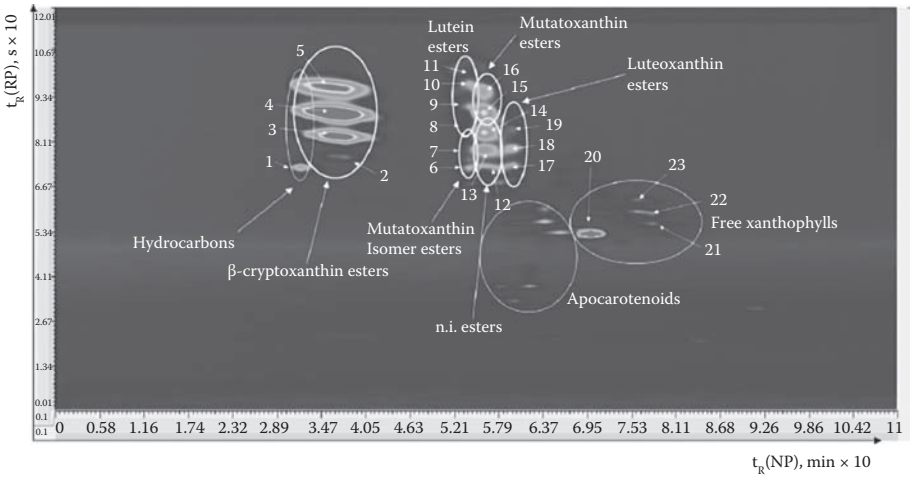

FIGURE 26.4 Contour plot of the comprehensive HPLC analysis of carotenoid esters present in mandarin essential oil. For experimental conditions and peak identification see ref. (Dugo, P., Herrero, M., Kumm, T., Giuffrida, D., Dugo, G., and Mondello, L., *J. Chromatogr. A*, 1189, 196–206, 2008. With permission.)

chromatography has been reported. The use of a mass detector along with diode array detection (DAD), after the separation on a chromatographic column, is also becoming a common approach in the analysis of carotenoid-containing samples [79].

26.4.5.1 Comprehensive LC × LC

Comprehensive two-dimensional NP (normal-phase)-RP HPLC with DAD has been applied for the complete resolution of free carotenoids from mandarin [4]. To increase the certainty in the compound identification, the method was further adjusted by adding mass spectrometric (MS) detection to the existing DAD. The analyzed constituents were first separated on a microbore silica column and orthogonally on a C_{18} column. In the first dimension the employed mobile phases consisted of *n*-hexane and ethyl alcohol in the linear gradient mode [4]. In the second dimension, the following mobile phases were used: 2-propanol and 20% water (v/v) in MeCN. Two different linear gradients were employed, one optimized for separation of hydrocarbons, the other for better resolution of xanthophylls. The effluent from the second column was split in two, as both detection systems, MS and DAD, were connected in parallel.

Comprehensive LC × LC has been also applied to analyze carotenoid esters [4]. The first separation was performed with a microbore cyano column, while the second-dimension separation was carried out on a C_{18} monolithic column. In the first dimension, the following mobile phases were used: *n*-hexane and *n*-hexane:butyl acetate:acetone (80:15:5, v/v/v). The second-dimension column as well as the mobile phases were the same as those described for free carotenoids, but the gradient was changed and optimized for separation of esters. The separation was performed in linear gradient mode. The separation results are presented in Figure 26.4. MS and DAD information were used to identify the analyzed esters, due to the lack of commercial standards. MS detection was of great help in the analysis of carotenoid esters, as many of them possess similar or even identical UV/Vis spectra, and thus DAD identification alone may be insufficient (see Figure 26.5). A further improvement in separation may be achieved with the application of C_{30} columns in the second dimension.

26.4.5.2 HPLC–MS

HPLC can be hyphenated with an MS detector to enhance the detection limit, as well as for fast and unambiguous structural assignment of carotenoids [80]. However, carotenoids are nonpolar substances, and so their ionization for nonspectrometric detection is difficult. Due to the lack of a

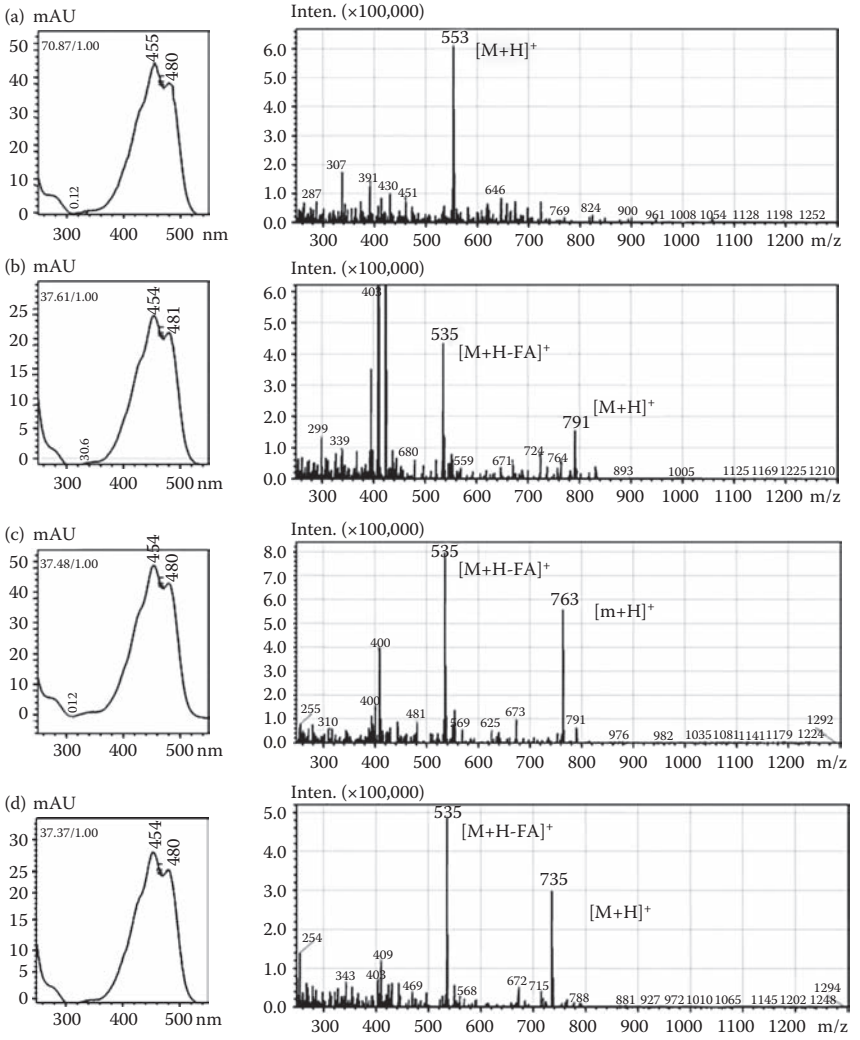

FIGURE 26.5 Example of the determination of different carotenoid esters according to the information provided by the two detectors. (a) β-Cryptoxanthin; (b) β-cryptoxanthin-C_{16} ester; (c) β-cryptoxanthin-C_{14} ester; (d) β-cryptoxanthin-C_{12} ester. (From Dugo, P., Herrero, M., Kumm, T., Giuffrida, D., Dugo, G., and Mondello, L., *J. Chromatogr. A*, 1189, 196–206, 2008. With permission.)

site for protonation, detection by electrospray ionization (ESI) is not simple. Despite the difficulties this mode of detection has been used following the LC separation on a C_{30} column. Better results are obtained by the use of additional chemicals that facilitate the ionization process, such as halogen-containing eluents [81,82], silver salts [83], or ferrocene-based derivatives [84]. For example, trifluoroacetic acid and heptafluorobutanol were added to enhance ionization of nonpolar carotenoids, for example, β-carotene. In this case the application of ESI-MS detection caused a 100-fold increase in sensitivity [81]. Ren and Zhang describe in detail the HPLC-ESI-MS identification of carotenoids from *Potamogeton crispus*. The authors present, in tabular form, the molecular ions and the mass fragments that might be useful in method development and carotenoid identification [18].

Due to the difficulties already mentioned, carotenoids have been investigated, more often, by the use of particle beam MS [85,86] or APCI (atmospheric pressure chemical ionization)–MS [87,88]. Breithaupt states that the positive mode of APCI-MS is a better choice for the analysis of carotenoids, as the sensitivity of all analyzed xanthophylls was 5–50 times higher when compared

to the negative mode [42]. Wingerath et al. separated 38 carotenoids and carotenoid esters by HPLC and utilized matrix-assisted laser desorption ionization post-source decay MS (MALDI-PSD-MS) in the identification of 11 carotenoids [89]. Mercadante et al. employed electron impact MS to confirm eight carotenoids in mango, 13 carotenoids in passion fruit and nine carotenoids in guava [90–92]. Coordination ion-spray (CIS)–MS was introduced by Strohschein et al. for the determination of a variety of unsaturated compounds such as carotenoids [83,93,94].

Thermal lens spectrometry (TLS) has been coupled with gradient HPLC for the complete resolution and identification of a complex carotenoid mixture [95]. The gradient HPLC separation was performed on a reversed-phase C_{18} column.

26.4.6 NP-HPLC

NP-HPLC has been applied only occasionally for the analysis of carotenoids, due the lack of resolving power when separating hydrocarbons and the more polar xanthopylls. In NP-HPLC carotenoids are separated into groups of different polarity according to the following elution order: hydrocarbons, monools, diols, and polyols. However, it was successfully used for the separation of the main carotenoid pigments of cereals, especially lutein and zeaxanhin [96]. A nitrile column has been applied for the separation of carotenoids in mango in a normal-phase system [97].

26.5 QUANTITATIVE ANALYSIS

The major carotenoids can be determined in plants either by open-column chromatography (OCC) or by HPLC [16]. The first approach is not currently recommended; however, reliable results obtained through its application has been reported in the literature [98,99]. The main problems of OCC are low sample throughput and results that depend heavily on the expertise of the analyst [16].

Carotenoids are normally quantified using an external calibration method [12]. The main problem in performing such analysis is the lack of proper commercial standards, and those available are not always pure enough for reliable results [16]. The highly unsaturated carotenoids are particualrly prone to isomerization and oxidation [16]. The quantification is always performed after saponification, because of the lack of proper carotenoid ester standards [4]. Usually, the following carotenoids have been quantified: lutein, zeaxanthin, lycopene, β-cryptoxanthin, α-carotene, and β-carotene [12]. Kimura and Rodriguez-Amaya were the first to quantify the amount of lactucaxantin in real samples [16]. Due to difficulties in complete separation of lutein and zeaxanthin, their amount is sometimes given as the sum of the content of both [100]. Li et al. state that xanthophylls and unidentified components in plant products are often not separated well, affecting the accuracy of quantitative determination of lutein and zeaxanthin [69]. The authors used a linear gradient HPLC method for the complete resolution and quantification of lutein and zeaxanthin in extracts of plant products. Müller reported a quantification method for carotenoids for which reference substances are not available [9]. Carotenoids were quantified by their 1% (1 cm) extinction coefficients, E. The fictitious concentration, c (=detector signal/E × 100), was related to the c value of β-carotene, whose actual concentration was known. Rare derivatives were quantified by the coefficient of the initial substance.

Kimura and Rodriguez-Amaya enumerated three ways of calculating of the carotenoid concentrations to compensate for changes in the detector's response [16]. These are (a) using full standard curves constructed on each day of analysis, (b) construction of full standard curves and recalibration on each day of analysis, and (c) use of response factors. However, most carotenoid papers do not specify the calculation method used.

Stock solutions are usually prepared by dissolving a proper amount of a standard (e.g., 1 mg) in an appropriate solvent in volumetric flasks. As the purity of all carotenoid standards is almost always less than 100%, the concentrations of the resulting solutions are calculated spectrophotometrically on the basis of E (1%, 1 cm), for example, lutein — 2551, ethanol, 445 nm; β-carotene — 2500, light petroleum, 451 nm; and lycopene — 2939, light petroleum, 474 nm [42]. For preparing

calibration curves, appropriate volumes of the respective stock solutions are diluted and subjected to HPLC analysis. The resulting peak areas are plotted against the concentrations.

The addition of a solvent modifier (TEA) to the mobile phase was shown to improve the recovery of carotenoids from the column [13]. Lauren et al. reported that the addition of *n*-decanol into the mobile phase prevented deterioration in column behavior caused by activation of residual silanols [101]. However, Epler et al. stated that the addition of *n*-decanol into the mobile phase did not caused any significant improvement in the recovery of carotenoids [102]. The authors reported that the addition of ammonium acetate also improves column recovery. The exact action of both TEA and ammonium acetate on carotenoid recovery is still unclear. Probably its effect is caused by buffering of the mobile phase or, more likely, of free silanols' acidity, as well as by prevention of reactions with free-metal ions [13]. Good recoveries of carotenoids from silica columns were reported by Kamber and Pfander, who added *N*,*N*-diisopropyl-ethylamine into the mobile phase [103].

The application of TLS detection, after the separation on a C_{18} column, provides lower limits of detection in comparison to UV/Vis detection [95].

Kimura et al. report that the concentrations obtained with C_{18} and C_{30} columns do not differ significantly for zeaxanthin, lutein, β-cryptoxanthin, and β-carotene in the all-*E*-configuration [26]. The authors state that all *Z*-isomers of the previously mentioned compounds are difficult to locate with the C_{30} column.

26.6 EXAMPLES OF HPLC ANALYSIS OF MEDICINAL PLANTS AND HERBAL PREPARATIONS CONTAINING CAROTENOIDS

The literature has generally concentrated on the qualitative and quantitative analysis of carotenoids in foods. However, there are many carotenoid-rich herbal medicines, whose biological activity can be at least partially attributed to these pigments. Several papers describe the application of HPLC in the analysis of plants used for preparing herbal drugs.

There are several reports on the analysis of the carotenoid content of *Calendula officinalis*. Baseline resolution of *cis* and *trans* isomers of lutein in saponified marigold extracts using a C_{30} column was reported by Delgado-Vargas and Pardes-Lopez [104,105]. High levels of lutein were found in the analyzed dried petals. Rivas identified four major esters of lutein in marigold extracts: dipalmitate, myristate–palmitate, palmitate–stearate, and dimyristate [106]. Bakó et al. report on the HPLC investigation of the carotenoid composition of the stems, leaves, petals, and pollen of *Calendula officinalis* L. [107]. In the petals and pollen, the main carotenoids were flavoxanthin and auroxanthin, while the stems and leaves mostly contained lutein and β-carotene.

Rosa canina is an herb commonly applied in traditional medicine. Its lipophilic fraction was proved to have antioxidative and anti-inflammatory properties [108]. HPLC analysis of saponified *Rosa canina* fruit extracts, on a C_{18} column, revealed that the major carotenoids were β-carotene, lycopene, β-cryptoxanthin, rubixanthin, zeaxanthin, and lutein [17]. The pair lutein and zeaxanthin remained unresolved under isocratic conditions, but their separation was somewhat better after the application of gradient elution. Machmudah et al. and Illes et al. investigated different factors influencing carotenoid extraction efficiency from rosehip fruit [29,109]. Both teams reported that the major carotenoids were lycopene, β-carotene, and lutein.

Due to their high anthocyanin as well as carotenoid content, *Vaccinium myrtillus* fruits have been often used in preventing age-related macular degeneration. Marinova and Ribarova reported that blackberries had the highest levels of carotenoids in comparison to other investigated berries [12]. The HPLC analysis of the these fruits revealed they were also characterized by the highest content of β-cryptoxanthin and β-carotene.

Carotenoids constitute one of the important groups of nutrients present in *Urtica dioica* L. Guil-Guerrero et al. used HPLC-MS to identify the most important carotenoids present in the leaves of this herb [70]. Nine carotenoids were identified, and lutein, lutein isomers, β-carotene, and

β-carotene isomers were the major carotenoids. The carotenoids were separated on a C_{30} column, and the components were eluted in order of decreasing polarity from polar oxycarotenoids to lipophilic hydrocarbons.

Breithaupt and Schlatterer analyzed selected dietery supplements used to delay age-related macular degeneration [71]. The authors used HPLC-APCI-MS to identify lutein and zeaxanthin in these supplements. These xanthophylls are structurally closely related, thus difficult to resolve completely. The lutein used in the production of dietary supplements is usually obtained from *Tagetes erecta* oleoresin, spinach, or broccoli sprouts, which is also a source of the free zeaxanthin form.

The carotenoid content of *Capsicum annum* fruits has been investigated by means of HPLC by Deli and Tóth [72]. In the chromatograms, 56 peaks were detected, and 34 carotenoids were identified. In the ripe fruits, capsanthin, capsorubin, zeaxanthin, cucurbitaxanthin A, and β-carotene were found to be the main carotenoids, the remainder being capsanthin 5,6-epoxide, capsanthin 3,6-epoxide, karpoxanthin, cucurbitaxanthin B, violaxanthin, cycloviolaxanthin, antheraxanthin, capsanthone, nigroxanthin, β-cryptoxanthin, and several *cis* isomers and furanoid oxides. In the unripe fruits the main carotenoids are lutein, zeaxanthin, and β-carotene.

An extract of dandelion (*Taraxacum officinale*) was analyzed by HPLC using a C_{30} column, as a result of which six geometric carotenoid isomers were separated [44]. The research revealed that all-*E*-lutein epoxide was the major carotenoid and that there were also high amounts of the (9*Z*)- and (9'*Z*)-isomers, although the latter may be an artifact.

Raju et al. describe an HPLC analysis of carotenoid-rich vegetables and plants that can be used for medicinal purposes, for example, in fighting vitamin A deficiency or age-related macular degeneration [110]. The carotenoid composition of chlorella tablets was investigated by Inbaraj et al. [111]. The authors performed HPLC analysis on C_{30} columns using gradient elution. All-*trans* lutein was present in an exceptionally large amount, which leads to the conclusion that the investigated tablets may be used to prevent age-related macular degeneration.

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27 HPLC of Steroids

Laurence Dinan, Juraj Harmatha, and René Lafont

CONTENTS

High performance liquid chromatography (HPLC) has become the method of choice for the separation, purification, identification, and quantification of phytosteroids. We briefly review the diversity and biological significance of the various classes of phytosterols, phytosteroids (brassinosteroids, bufadienolides, cardenolides, ecdysteroids, steroidal saponins, steroidal alkaloids, vertebrate-type steroids, and withanolides), and steroid-related triterpenoids (cucurbitacins). We summarize the HPLC systems (predominantly reversed-phase [RP]) that have been applied to the separation of these molecules and consider how the potential of HPLC is being extended for identification and dereplication by coupling the chromatographic separation (off-line or on-line) to spectrometric (infrared spectroscopy [IR], mass spectrometry [MS], nuclear magnetic resonance [NMR]) or biological methods (bioassays, immunoassays), or even a combination of several of these. In view of the plethora of analogues for each class of phytosteroid found in plants and their diverse and manifold important biological activities, this area of research can be expected to continue to develop significantly over the coming years.

27.1 INTRODUCTION AND SCOPE OF THE REVIEW

27.1.1 Usefulness and Validity of HPLC for Sterols and Steroids

The phytosteroids present a wide range of polarities, especially when one takes into account the existence of polar and nonpolar conjugates and the possibility of charged forms (e.g., steroidal alkaloids or sulfate esters of ecdysteroids or cardenolides [1,2]). HPLC provides a wide range of high-resolution phases, mobile phases, and modifications (e.g., use of ion-pair reagents, cyclodextrins, etc.), such that any complex combination of steroids can be resolved by sequential separations on two or three judiciously chosen systems. Further, the availability of different size columns from nano- and micro- through analytical to preparative, together with appropriate delivery systems, allows HPLC at all levels, such that with microbore HPLC one achieves sensitivities comparable with gas chromatography (GC) — often without the need for derivatization — whereas preparative HPLC is replacing open-column chromatography because of its rapidity, flexibility, and reproducibility. A wide range of HPLC detectors (ultraviolet/visible [UV/Vis], evaporative light scattering detector [ELSD], etc.) permit the ready and sensitive detection of most steroids. The scope for identification and dereplication is being extended by the use of coupled systems (on- and off-line) to give high information output, often resulting in unambiguous identification of eluting compounds based on retention time, spectral (UV/Vis, IR, NMR, MS) characteristics, and/or biological properties (antibody recognition, etc.). The diversity of applications to which HPLC contributes in the analysis of phytosteroids is extensive:

- Analysis and quantification of individual components
- Purification of individual or multiple components
- Dereplication
- Identification of novel analogs
- Determination of the distribution within plants
- Comparative studies (including chemotaxonomic studies)
- Quality control of pharmaceutical/nutraceutical preparations
- Monitoring the levels of secondary products in genetically modified organisms (GMOs)
- Quantitative structure-activity relationship (QSAR) correlations of polarity and biological activity

27.1.2 Scope of the Review

This chapter updates our previous, more general chromatographic review [3], focusing on the application of HPLC to the separation and identification of the various classes of phytosteroids and complementing our earlier review on the thin-layer chromatography (TLC) of phytosteroids [4]. A certain emphasis will be placed on ecdysteroids, not only because this reflects a main interest of the authors, but also because HPLC separations of this class of steroid have been investigated more systematically [3]. Since HPLC has essentially become routine for phytosteroids, we will, in addition to briefly giving examples of the separation systems for each class and the applications to which HPLC has been put, emphasize the recent developments in coupled techniques and briefly indicate the varied medical and pharmaceutical applications of phytosteroids. Representative structures of members of each of the chemical classes considered are shown in Figure 27.1.

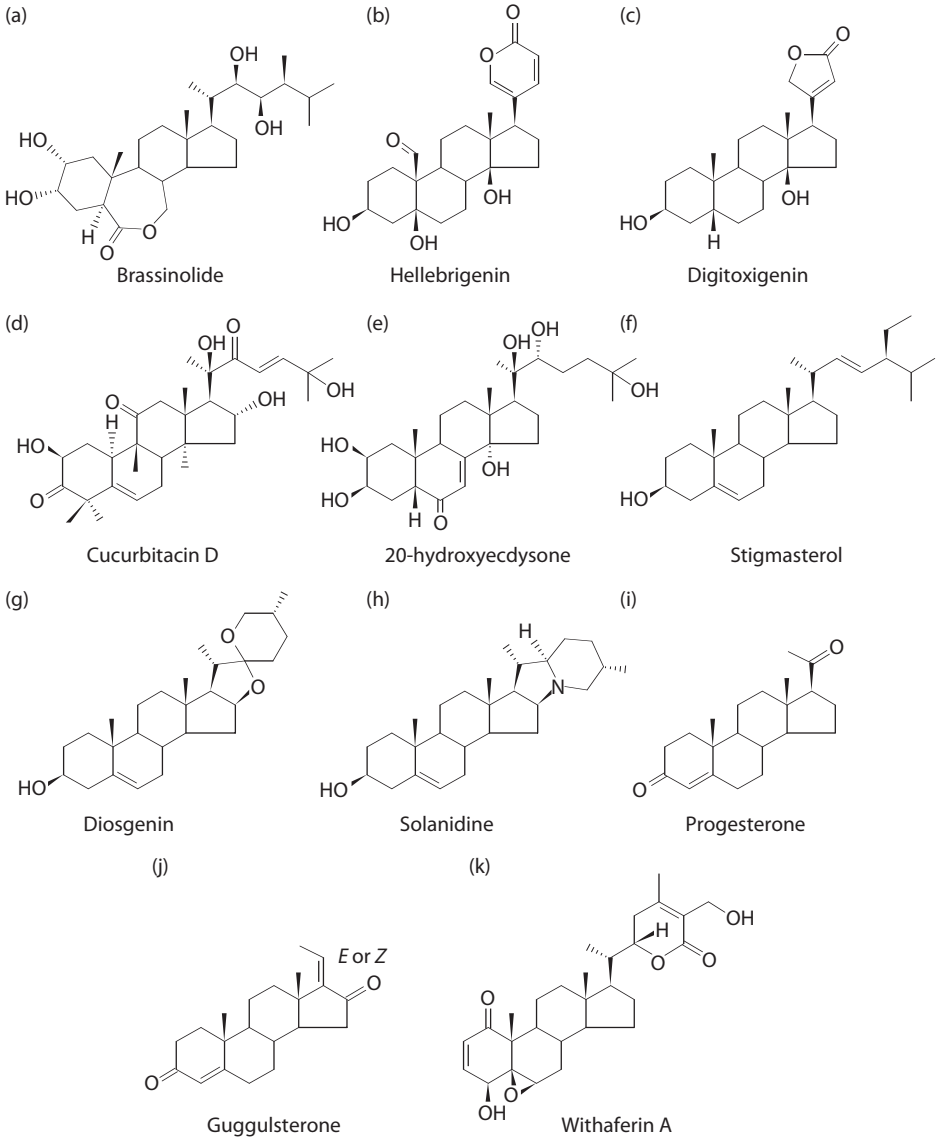

FIGURE 27.1 Structures of representative members of each of the phytochemical classes considered. The configurations are 8β-H, 9α-H and 14α-H, unless otherwise depicted.

27.2 SURVEY OF THE LITERATURE CLASS BY CLASS

27.2.1 Brassinosteroids

27.2.1.1 Structural Diversity and Biological Significance

Brassinosteroids are derivatives of 5α-cholestane with two vicinal diols (2α,3α and 22*R*,23*R*), a 6-keto or a 6-oxalactone group in ring B and various substituents in position 24 (24*R*/*S*-methyl, 24-methylene, etc.). Their occurrence seems to be general in the plant kingdom. Over 50 natural brassinosteroid analogs have been identified [5], including brassinolide (Figure 27.1a). Brassinosteroids are growth-promoting steroid hormones in higher and lower plants. Several authors have also suggested that brassinosteroids might interfere with the action of ecdysteroids, with which they share a superficial resemblance, in insects by acting as ecdysteroid receptor agonists or antagonists (summaried in [6]), but activity is seen only at concentrations too high to be attainable in nature [7].

27.2.1.2 Separation Systems and Spectroscopic Detection

The absence of a significant UV/V is chromophore in brassinosteroids, together with their very low concentration in most plant material, delayed the application of HPLC as a method of analysis for this class of compounds, in favor of GC-MS after derivatization, which provided the necessary sensitivity and specificity. However, a method for the quantitative precolumn derivatization of the 2α,3α- and 22,23-diols as the bis-*m*-aminophenylboronate, together with RP-HPLC separation and fluorimetric detection by a postcolumn reaction with *o*-phthalaldehyde and cyanide ions of the 24-epimers of brassinolide and castasterone, has been developed [8]. Also, LC-MS with atmospheric pressure chemical ionization (APCI) has been used for the identification and analysis of brassinosteroids as their naphthaleneboronates after separation at 45°C on a C_{18} column eluted with acetonitrile (ACN)–H_2O (9:1, v/v) [9]. Generally, HPLC of brassinosteroids is performed on C_{18} columns, eluted with 65–80% ACN–H_2O mixtures. In addition to the identification of brassinosteroids in plant extracts (e.g., Ref. [10]), HPLC has contributed to the investigation of the biosynthesis, interconversion, and metabolism of brassinosteroids in plant cells and differentiating cultures. However, GC-MS remains the method of choice for quantification (e.g., Ref. [11]).

27.2.2 Bufadienolides

27.2.2.1 Structural Diversity and Biological Significance

Bufadienolides are usually C_{24} steroids and their glycosides. They possess a chromophoric six-membered lactone (α-pyrone) ring at C-17β, which imparts a characteristic UV absorbance (λ_{max} at 296–299 nm in methanol [$\varepsilon = 5000$ L $mol^{-1}cm^{-1}$] and a second absorption at 200–220 nm). Many possess a 5β-hydroxyl (A/B-*cis* ring junction), a *trans*-B/C ring junction, a 14β-hydroxyl (C/D-*cis* ring junction), and an aldehyde group at C-19 (e.g., hellebrigenin; Figure 27.1b [12]). Bufadienolides occur in both plants and animals, and over 160 analogs have been identified from members of six plant families (Crassulaceae, Hyacinthaceae, Iridaceae, Melianthiaceae, Ranunculaceae, and Santalaceae) [13]. Helleborin was previously used to treat heart arrhythmias, but bufadienolides possess many other biological activities, ranging from antitumor activity [14] to toxicity to mammals [12] and insects [15]. Some are extremely potent inhibitors of T-cell activity, raising interest in their immunosuppressive potential [16]. Evidence has been obtained, as with the cardenolides, that suggests the existence of endogenous bufadienolides in mammals (e.g., Ref. [17]).

27.2.2.2 Separation Systems and Spectroscopic Detection

In comparison to the other class of cardiac steroids (cardenolides), the bufadienolides have received much less study. It is generally assumed that the biological activities of the two groups are similar, although this may not be valid [16]. As examples of the use of HPLC in the isolation and

identification of bufadienolides from plant sources, one can cite two relatively recent studies on *Helleborus* spp. Meng et al. isolated two new and one known bufadienolides and two ecdysteroids from seeds of *H. torquatus* [18]. The bufadienolides were isolated by sequential HPLC on a RP(C_6) semipreparative column eluted with 35% methanol (MeOH) in water and a normal-phase (NP) DIOL semipreparative column eluted with 6% MeOH in dichloromethane. Watanabe et al. used preparative RP(C_{18})-HPLC, eluted with ACN–H_2O (3:7, v/v), to isolate one new and two known bufadienolide glycosides from the rhizomes of *Helleborus orientalis* [19]. Preparative HPLC was also used in the purification of 52 bufadienolide analogs for an impressive investigation of the structure-activity relationship of the T-cell suppressive effect of these compounds; proscillaridin A (from *Urginea* sp.) was identified as the most active compound, being 16,384-fold more potent than cortisol [16].

27.2.3 Cardenolides

27.2.3.1 Structural Diversity and Biological Significance

The cardenolides are structurally closely related to the bufadienolides but possess a five-membered lactone (butenolide) ring at C-17β. This imparts a characteristic UV absorption at ca. 220 nm in MeOH. The A/B- and C/D-ring junctions are *cis*-fused with a 14β-hydroxyl and a 5β-H (e.g., digitoxigenin; Figure 27.1c). Cardenolides and cardenolide glycosides are widely distributed in the plant world but are particularly associated with the Asclepiadaceae, Scrophulariaceae, Ranunculaceae, and Convallariaceae. Cardenolide sulfates are present in *Adonis aleppica* (Ranunculaceae) [2]. Digoxin and ouabain are significant inhibitors of mammalian Na^+/K^+-ATPases [20]. Evidence is accumulating that plant cardenolides (and other cardiotonic steroids: bufadienolides) mimic endogenous compounds in vertebrates in binding to a highly conserved site on the Na^+/K^+-ATPase [21]. In addition to their traditional pharmacological applications in controlling sodium balance and blood pressure in disease states related to cardiac insufficiency, the cardenolides also have anticancer activity [21] and deter insect feeding and oviposition [22].

27.2.3.2 Separation Systems and Spectroscopic Detection

RP(C_{18})-HPLC with a complex ACN–H_2O gradient from 27.5 to 70% ACN and monitoring at 220 nm has been used to examine the chemo-ecological relationship between the cardenolide levels and profiles in monarch butterflies (*Danaus plexippus*) and those of the larvae's host plant, *Asclepias fruticosa* (milkweed), allowing the separation of 28 cardenolides present in the latex of *A. fruticosa* [23]. A highly efficient and sensitive RP-HPLC method for the separation of a large number of cardenolide analogs present in *Digitalis lanata* leaves has been reported [24]. The C_{18} column was eluted at 40°C with an ACN–H_2O gradient from 20 to 55% ACN and monitored at 225 nm. In view of the pharmacological importance of the cardiac glycosides from the leaves of *D. lanata* and the dependence of their profile and levels on the time of harvesting, fungal infection, etc., an RP-HPLC method has been proposed for the quality control of lanatoside C (a major primary glycoside) levels in preparations from this source [25]. Braga et al. employed a micro-HPLC separation (on a C_{18} 100 × 2.1 mm ID column, eluted at 40°C and 0.2 mL/min with a multistage gradient of ACN in H_2O and monitored at 220 nm) for the quantification of 10 major cardenolides in Brazilian plants of *D. lanata* at two stages of development in order to identify and select higher-yielding plants for improvement of the cultivar [26]. Ankli et al. used RP(C_{18})-HPLC, eluted with ACN–H_2O mixtures, for the final purification of five cardenolides from roots of *Crossopetalum gaumeri* (Celastraceae) [27].

27.2.4 Cucurbitacins: Steroid-Related Triterpenoids

27.2.4.1 Structural Diversity and Biological Significance

The cucurbitacins are a group of highly oxygenated C_{30} triterpenoids possessing a 19(10→9β)-abeo-10α-lanost-5-ene skeleton (including a gem-dimethyl group at C-4 and further methyls at C-9, C-13,

and C-14). All cucurbitacins also possess a 5(6)-double bond (e.g., cucurbitacin D; Figure 27.1d). Many cucurbitacins absorb UV light with a maximum at ca. 230 nm in MeOH or ethanol [28], owing to the presence of chromophoric α,β-unsaturated ketones in the A ring and side chain, although other analogs exist that have a maximum at a higher wavelength or no maximum above 210 nm. The cucurbitacins are most associated with the Cucurbitaceae but have also been identified in members of other plant families (Begoniaceae, Cruciferae, Datiscaceae, Desfontainaceae, Elaeocarpaceae, Polemoniaceae, Primulaceae, Rosaceae, Rubiaceae, Scrophulariaceae, and Sterculiaceae). Cucurbitacins B and D occur most commonly, but about 50 other analogs (including glycosides) have been characterized [29]. Cucurbitacins taste extremely bitter and are known for their toxicity [30]. Their cytotoxicity led to them being considered as anticancer agents (e.g., [31]), but their toxicity proved too general [32]. The biological effects of cucurbitacins include antifeedant activity against most animals, feeding attraction for diabroticite beetles [33], and antagonist activity at the ecdysteroid receptor of insects [34]. Their pharmacological effects on mammals are wide ranging, including purgative, anti-inflammatory, and antifertility effects [32], and they possess antioxidant properties [35].

27.2.4.2 Separation Systems and Spectroscopic Detection

Curcurbitacins from plant sources have been purified by NP-HPLC (e.g., [36]) and RP-HPLC (e.g., [37,38]), whereas cucurbitacin glycosides are usually purified by RP-HPLC (e.g., [39]) for identification. Owing to the bitterness associated with cucurbitacins and their pharmacological properties, HPLC methods have been developed for their reliable quantification and the quality control of foodstuffs and pharmacological preparations. These methods generally use RP(C_8 or C_{18}) columns, eluted with MeOH or ACN in water for free cucurbitacins (e.g., [34,40–42]) or more complicated mobile phases for cucurbitacin glycosides (e.g., ACN–MeOH–50mM NaH_2PO_4 in H_2O [43]). In an interesting application, RP(C_{18})-HPLC with gradient elution using MeOH (60–75%) or ACN (30–70%) in water has been used to establish a clear relationship between the chromatographic hydrophobicity index for 18 cucurbitacins and cucurbitacin glycosides and their cytotoxicity toward human HepG2 cells [44].

27.2.5 Ecdysteroids

27.2.5.1 Structural Diversity and Biological Significance

Ecdysteroids represent a very large family of steroids (more than 400 representatives) found in both animals and plants [45]. Among these, phytoecdysteroids comprise more than 300 closely related molecules that have been isolated and identified from diverse plant sources, ranging from algae to ferns to angiosperms (monocots and dicots). Phytoecdysteroids are predominantly C_{27}, C_{28}, or C_{29} steroids related to 20-hydroxyecdysone (20E: Figure 27.1e), differing in the number of carbon atoms and the number and position of substituents but sharing a specific 14α-hydroxy-7-en-6-one chromophore (λ_{max} at 242 nm in MeOH or 247 nm in water, and ε_{max} = ca. 12,000 $L.mol^{-1}.cm^{-1}$), which allows their HPLC detection in the low nanogram range. They are typically characterized by the presence of several hydroxyl groups at positions 1, 2, 3, 5, 11, 12, 14, 20, 22, 25, or 26, which gives ecdysteroids considerable water solubility. Polar (glycosides) and nonpolar (acetates) conjugates also occur frequently in plant extracts. Ecdysteroid-containing plants generally possess one or a few major ecdysteroid analogs (predominantly 20E and polypodine B [polB]), together with a "cocktail" of minor ecdysteroids [46]. A wide range of (largely positive) pharmacological effects on vertebrates have been reported: adaptogenic, anabolic, hepatoprotective, hypocholesterolemic, hypoglycemic, and wound-healing (reviewed in Ref. [47]).

27.2.5.2 Separation Systems and Spectroscopic Detection

Ecdysteroids have a wide range of polarities, since the number of hydroxyl groups usually ranges from 4 to 9, and they often occur as conjugates with polar (sugars) or nonpolar (acetic acid,

benzoic acid, etc.) moieties. Differences between analogs may also be more subtle, concerning, for example, the position or even just the stereochemistry of the hydroxyl groups, which complicates ecdysteroid separation. As a consequence, obtaining pure molecules from plant extracts, which generally contain a complex cocktail of ecdysteroid analogs (e.g., [48,49]), generally requires a combination of several (two to three) HPLC systems [3,50–52]. Both NP- and RP-HPLC can be used for ecdysteroid separation, and many different systems have been developed that provide different selectivities [53,54].

General NP- and RP-HPLC Systems

Many different HPLC systems have been developed for the separation of ecdysteroids [3,50,53,55]. Ecdysteroids are amenable to both NP- and RP-HPLC. When compared to TLC, the choice of solvents in HPLC is limited by their UV cut-off, which does not allow the use of, for example, ethyl acetate, acetone, or aryl hydrocarbons. NP-HPLC on silica (or diol-bonded) columns requires rather polar mobile phases that contain significant amounts of an alcohol (MeOH, ethanol, propan-2-ol) and even some water in a hydrocarbon or a chlorinated base solvent (e.g., cyclohexane, dichloromethane, and dichloroethane). Thus, dichloromethane–propan-2-ol–water or cyclohexane–propan-2-ol–water mixtures have been extensively used [51,53]. The proportion of alcohol can be adjusted over a wide range, and these systems can even be used with the polar ecdysteroid glycosides [56]. Diol-bonded columns allow gradient systems to be used [57]. RP-HPLC systems use MeOH, ACN, propan-2-ol, or tetrahydrofuran mixed with water (possibly containing 0.1% [v/v] trifluoroacetic acid [TFA]) under isocratic or gradient conditions. They are used more widely than NP systems. In RP systems, conjugation with a sugar results in a surprisingly limited increase of polarity (that is, a small reduction of the retention time) with hexoses (glucose, galactose), which becomes insignificant with a pentose, and may even result in a decrease of polarity in the case of deoxy-sugars [49,56].

The HPLC behavior of certain ecdysteroids is sometimes unexpected. Thus, side-chain cleavage products (poststerone, rubrosterone) behave as if they were polar in RP-HPLC and nonpolar in NP-HPLC. When analyzed by NP-HPLC, ecdysteroids bearing a lactone on their side chain (e.g., cyasterone or sidisterone) behave like nonpolar compounds with dichloromethane-based solvents and like polar ones with cyclohexane-based solvents [53]. Similarly, polypodine B (5β,20-dihydroxyecdysone) behaves very differently with these two NP-HPLC solvents [54]. Thus, selectivity is an important parameter in HPLC analysis of ecdysteroids [53], and it is quite difficult to predict the HPLC behavior of a given ecdysteroid just by comparing its structure to that of the reference compound 20-hydroxyecdysone. An example of a separation of ecdysteroids by RP-HPLC is given in Figure 27.2.

Diversity of HPLC Separation and Detection Methods

HPLC techniques have been used for both analytical and preparative purposes. (Semi)preparative columns (9.4–23.2 mm ID) offer similar efficiencies to analytical ones, and they can be used to isolate amounts suitable for full chemical identification and biological activity determination (e.g., [58]). In contrast, short small-bore columns reduce solvent consumption and provide an increased sensitivity of detection, and their resolution is often sufficient to separate the compounds of interest from impurities.

In order to improve the separation of some compounds, specific HPLC techniques have been developed. Thus, an efficient RP-HPLC separation of 20-hydroxyecdysone and polypodine B can be obtained by using a β-cyclodextrin-bonded stationary phase [59]. Conversion of the 20,22-diols into boronic esters may be used to change the chromatographic properties of ecdysteroids that possess such a diol [60,61].

The conversion to fluorescent derivatives using 1-anthroyl-nitrile has been proposed in order to increase the sensitivity of detection [62], but the use of this technique has been restricted to insect materials, where ecdysteroid concentrations are far lower than in ecdysteroid-containing plants.

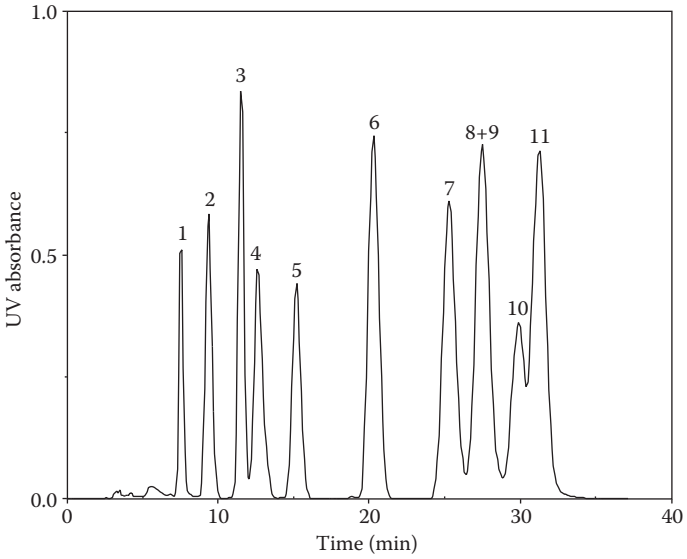

FIGURE 27.2 Separation of an ecdysteroid mixture by RP-HPLC. Operating conditions: column: Spherisorb 5ODS2, 250 mm long, 4.6 mm ID; solvent: methanol–water (35:65, v/v), flow rate 1 mL.min^{-1}; detection UV 254 nm. 1, (17β-H)dihydrorubrosterone; 2, 20,26-dihydroxyecdysone; 3, rubrosterone; 4, turkesterone (11α,20-dihydroxyecdysone); 5, integristerone (1β,20-dihydroxyecdysone); 6, abutasterone (20,24-dihydroxyecdysone); 7, polypodine B (5β,20-dihydroxyecdysone); 8, 20-hydroxyecdysone; 9, poststerone; 10 and 11, 20*R*- and 20*S* epimers of dihydropoststerone.

Such a technique could be of interest in the case of phytoecdysteroids lacking the 7,8-double bond (e.g., [63]), which do not absorb UV at ca. 245 nm. Alternatively, such ecdysteroids could be detected using a universal detector like ELSD.

Many detection techniques have been investigated for the HPLC analysis of ecdysteroids. Diode array detectors (DAD) have now become conventional (e.g., Refs. [64,65]); they allow in-line access to absorbance spectra, which may be of interest to decide whether a UV-absorbing peak may correspond to an ecdysteroid or to characterize some specific classes of ecdysteroids conjugated to an aromatic moiety [49]. Fourier-transform infrared (FT-IR) detectors, mass spectrometry, and other hyphenated detection methods will be considered in Section 27.3.

27.2.6 Phytosterols

27.2.6.1 Structural Diversity and Biological Significance

Phytosterols belong to the large family of phytosteroids, structurally related steroid metabolites of plant origin. The close structural similarity of phytosterols indicates their joint biosynthetic origin, thus evoking also a close biogenetic relation. In a broader sense, they are isoprenoid constituents that are biosynthetically most closely related to triterpenoids [66]. In particular, they are also biosynthetic precursors to the majority of phytosteroids (the more oxidized specific steroids in plants, also covered in this chapter), as well as to a series of essential steroids in other coexisting organisms, for example, insect ecdysteroid hormones [67].

The sterols comprise several major groups characterized by the presence of the typical steroid (androstane) skeleton and by a hydroxyl group at C-3, normally in the β-configuration. Moreover, members of the phytosterol family possess a branching side chain of 8 to 10 carbon atoms at C-17. Variation in the side chain forms the basic structural diversity and thus also the basis for the classification of phytosterols. The simplest is the cholestane (C_{27}) type, next is the C-24 methyl homologue

ergostane (C_{28}) type and its C-24 epimer campestane, and then comes the C-24 ethyl homologue stigmastane (C_{29}) type and again its epimer poriferastane. All types can contain one to three double bonds in several positions of the sterane skeleton or in the side chain (see, e.g., stigmasterol in Figure 27.1f). This increases the number of basic types. Each type can occur also in C-5 epimeric form, which doubles the number of types. A number of combinations of the parent hydrocarbon ring and side chain have been nominated by IUPAC-IUB Nomenclature of steroids [68] for classification and nomenclature purposes, but for practical use the previously introduced and well-established trivial names are preferred [69].

Plants often contain phytosterols in various conjugated forms, which even further enhances structural diversity. The most frequent are steryl fatty acid esters, phenylpropanoate steryl esters, steryl glycosides, and acylated steryl glycosides [70]. Their structural diversity and phylogenetic distribution in plants and plant organs are reviewed by Moreau et al. [71].

Phytosterols' biological functions and bioactivities are as extensive as are their structural variations. Sterols regulate the fluidity of cell membranes and play a role in cellular differentiation and proliferation. They influence seed germination and participate in some signal transduction events as well. Steryl conjugates serve as transport and storage components [72]. The biological functions and effects of sterols also determine their various pharmacological or nutraceutical activities [73–78].

27.2.6.2 Separation Systems and Spectroscopic Detection

Plants regularly contain nonconjugated free phytosterols in complex mixtures with β-sitosterol and/or stigmasterol as the major components accompanied by several minor compounds, such as campesterol, fucosterol, and other homologues or isomers [79]. These constituents are not easily separated. They readily crystallize together, and they do not separate from each other by common chromatographic techniques (countercurrent chromatography [CCC], TLC). Formerly, chemical transformations were applied for separation; for example, trifluoroacetylation improved separation significantly [80]. In special cases, argentation techniques (using $AgNO_3$-coated silica) have also been applied [81]. Often, the low-polarity chromatographic fractions (containing free phytosterols) were contaminated by *n*-alkanes and *n*-alkanols, which promote the biological activity of phytosterols [82] but do not help their identification.

For isolation of single sterols or conjugates, solvent extractions with hexane, methylene chloride, or chloroform are generally used. In the case of conjugates, saponification of samples precedes the chromatography in order to obtain the total sterol content [79]. Alternatively, the plant samples can be extracted by supercritical fluid extraction [83,84]. Preliminary partial separation (fractionation) used to be achieved by simple column chromatography or by preparative TLC. Both methods are suitable only for sample preparation, preliminary separation (crude purification), qualitative detection, or assays detecting (estimating) the sterol content. HPLC separation of sterols can be performed by either NP- or RP-HPLC, but in no case will these techniques provide an efficiency comparable to that achieved by GC.

NP-HPLC has been of limited use for free sterol analysis, owing to its rather poor selectivity, but there are a few reports on its use for steryl conjugates. We just mention here the method developed by Nyström et al. [85] using a diol-bonded column: Steryl glycosides were analyzed using an isocratic mixture of hexane–acetic acid (1000:1, v/v) and isopropanol (85:15, v/v) with ELSD, whereas the less polar steryl ferulates were analyzed with a mixture of hexane–isopropanol–acetic acid (99:10:0.1, v/v/v) and UV (315 nm) detection.

RP-HPLC can be performed using MeOH–H_2O (9:1 or 95:5, v/v) or ACN–propan-2-ol–H_2O (2:1:1, v/v/v), with the latter allowing efficient separation of Δ^5- and Δ^7-sterols [86]. A gradient of ACN in H_2O (+0.01% acetic acid) also proved efficient [87]. For quantitative analysis GC-MS and/or HPLC-MS techniques are required. Reports concerning application of these techniques in lipid analyses are well-reviewed by Abidi [79] and more generally by Goad and Akihisa [69]. However, even such hyphenated techniques necessarily require confrontation with individual authentic standard sterols, while for the more suitable GC-MS analytic method, computer databases (spectral

libraries) are now available. Recently, many reports from well-equipped laboratories offer valuable structural information (in the form of diagnostic ions obtained, e.g., by HPLC-ion trap MS/MS); structural elucidation can then be achieved by comparing results with published spectral data or with data obtained by direct analysis of well-identified standard samples [83,84,87–92]. Through the introduction of special separation facilities (columns with capillary electrochromatographic [CEC] stationary phases) it was possible to analyze pairs of sterol 24-epimers, and also the campesterol–stigmasterol pair, using a C_{18} column with an aqueous mobile phase [93–95].

27.2.7 Steroidal Saponins

27.2.7.1 Structural Diversity and Biological Significance

Steroidal saponins, together with the closely related triterpenoid saponins, are a widely distributed class of secondary metabolites in plants and marine organisms. From the chemical-structural point of view, they are glycosidic derivatives of mono- or multi-hydroxylated steroid compounds possessing one or more oligosaccharide chains (mono-, di-, or tri-desmosides) linked to a steroid aglycone with specific skeletal features and typically localized substituents. The sugar chain is usually attached to C-3 (less frequently to C-1, C-2, C-6, or the side-chain carbons) and contains predominantly two to five linear or branched monosaccharide units. The classical definition of saponins is based on their surfactant or detergent properties (*sapon* means "soap"), reflected in their stable foam formation in water solutions. This arises because they contain both water-soluble and fat-soluble molecular moieties. These specific structural features determine in many respects the main physicochemical properties of saponins. Their rather high solubility in water and high polarity in organic solvents caused many problems in earlier analytical and preparative examinations. However, these same properties are now convenient for methods based on HPLC and, more recently, for the rapidly developing HPLC-hyphenated approaches.

Steroidal saponins occur frequently, with wide distribution, in the plant kingdom, both in a qualitative and a quantitative sense. It is known that over 90 plant families contain saponins [96], and many new occurrences are still being reported [97]. The saponin content depends on many factors, such as the cultivar, the age, and the geographic locality of the plant. Considerable variation (mainly quantitative) can be observed in organs [98]. High contents are associated mostly with reproductive organs (flowers, seeds [99]) or lateral roots (root hairs), medium levels with stems and leaves, and low levels with roots or bulbs. Steroidal saponins are less widely distributed in nature than the more frequent triterpenoid-type saponins. The main sources of steroidal saponins are species in the Liliaceae, Dioscoreaceae, and Agavaceae, mainly the genera *Allium*, *Asparagus*, *Lilium*, *Agave*, *Yucca*, and *Dioscorea*.

Steroidal saponins of plant origin can be divided into two main groups. The largest group is the spirostanol glycosides, comprising aglycones of the spirostane type (e.g., diosgenin; Figure 27.1g) with a sugar chain generally situated in position C-3. For spirostanols, it is typical that the spiroketal arrangement is linked at C-22. Structural variations of spirostanols depend mainly on the stereochemistry at position C-5, with a $5\tilde{\alpha}$H or $5\tilde{\beta}$H configuration (*trans* or *cis* A/B-ring annelation, respectively), and at position C-25, with the methyl group in *R*- or *S*-configuration. The presence of a double bond in position 5(6) or 25(27) increases the variability, which can be further multiplied by the presence of one to four hydroxyls at almost any position but mainly at 1, 2, 3, 5, 6, 11, 12, and 15 (and again in $\tilde{\alpha}$ or β-configuration). Over 45 spirostanol-type aglycones have been structurally identified [96,100] and, when taking into consideration also the oxidized forms (usually carbonyls at positions 6 and 12, or a lactone forming carbonyl at C-26), then there are over 100 analogs [101]. The number of structural types rises still further with the variable sugar chains linked to the hydroxyl at position C-3 [102].

The second typical group comprises furostanol glycosides with aglycones having a spirostanol-like skeleton but with an open side chain (transformed ring F by hydrolysis of C-22 acetal to 22,26-diol). Fewer furostanol aglycone structural forms have been identified than for spirostanols, but more

glycoside conjugates (saponins) exist, as the sugar chains can be linked not only to position C-3 but often also at C-26. Furostanols can be transformed during enzymatic hydrolysis to spirostanols, and under certain conditions spirostanols can also be transformed by acid hydrolysis to furostanols. Moreover, etherification at position C-22 can also occur (i.e., formation of methyl ethers during methanol extractions), or dehydration of the hydroxyl at position C-6 can form 6(7)-ene derivatives. Such side reactions can form many artifacts, which have to be taken into consideration when selecting methods for saponin analysis or separations.

A small and unusual group of steroidal saponins are osladin and polypodosaponin [103–105] and the polypodosides A, B, and C [106,107] with open ring E and a preserved six-membered hemiacetal ring F, as well as sugar chains linked to both C-3 and C-26 hydroxyls. These saponins are responsible for the very sweet taste of *Polypodium vulgare* (sweet flag) and *P. glycyrhiza* (licorice fern). The specific 6-keto-7-ene moiety in their structure classifies these compounds among the ecdysteroids as well [45]. Not only their structural relation but also their co-occurrence with the major phytoecdysteroids (polypodine B and 20-hydroxyecdysone) in *P. vulgare* indicates a biogenetic relationship between these compounds. Another unusual group consists of C_{21} steroids with modified pregnane and 13,14; 14,15-disecopregnane skeleton types; these are the main components in the Asclepiadaceae family [108]. These compounds are considered to possess cytotoxic, antimicrobial, herbicidal, and anti-inflammatory activities [109,110].

Saponins often occur in plants that are used in human and animal nutrition [111], in traditional or modern medicine [112], or as herbal remedies in food, pharmacy, cosmetics, and so on [113], or other commercially important preparations and products [96,114]. For such purposes they are produced on a large scale using general or specially modified separation processes (e.g., the formation of water-insoluble complexes with cholesterol). The investigation of a wide variety of biological and pharmacological activities [109], chemoecological activities in plant–insect interactions [98], or other antimicrobial, molluskicidal, antifertility, and immuno-modulatory activities and physiological functions [115] often requires several specially adapted analytic and separation processes.

27.2.7.2 Separation Systems and Spectroscopic Detection

Steroidal saponins are usually extracted from dry or fresh plant material with aqueous MeOH. After evaporating a major part of the MeOH, the water portion is partitioned with ethyl acetate (to remove nonpolar constituents) and then partitioned with *n*-butanol to extract saponins (together with other polar constituents) and remove sugars, salts, and other highly water-soluble components. After removal of the solvent, the saponins can be separated by open-column chromatography on silica with a gradient solvent system of chloroform–MeOH–H_2O (87:12:1–14:6:1, v/v/v), or by low-pressure column chromatography using coarse-grained silica gel RP C_{18} with a gradient solvent system of MeOH–H_2O or ACN–H_2O. In some cases, especially for the first-step of crude extract separation, application of Sephadex LH-20 with MeOH or a suitable adjusted isocratic MeOH–H_2O solvent system seems the most useful. An extensive list of columns used for low-pressure column chromatography separations of saponins from particular plant sources, as well as various solvent systems applied to these separations, is provided in several reviews [96,116,117]. For successful separation of saponin mixtures, the stationary and mobile phases have to be selected carefully (and assessed experimentally). In some cases a combination of various phases has been used, such as a first-step fractionation on a short RP-18 column (or on a Sephadex LH-20 column), followed by separation into individual saponins on silica gel columns. Final purification is nowadays accomplished using HPLC systems with normal- or RP columns, especially if only low amounts of material are available [108,118–121].

Saponins' highly polar nature and rather high molecular weight, as well as their close structural similarities (isomers or epimers of the aglycone or sugar parts), has often caused difficulties in routine chromatography separations. TLC and simple column chromatography, as used for several decades in the analysis (detection, separation) of saponins, suffered mainly from low resolution [3,4,116]. HPLC, with its much greater resolution and the possibility of various quantitative

detection techniques, has become the leading method of saponin analysis. A large number of HPLC analyses employing various columns and solvent systems are summarized and commented on in the review by Oleszek and Bialy [117]. The only difficulty is the absence of a suitable chromophore for UV detection. Most published data are based on HPLC profiles recorded at 200–210 nm. This range, however, allows only nonspecific detection and thus greatly reduces the use of this technique to quantify saponins in unseparated samples. To overcome this problem, ELSD has been applied. There are already over 50 papers reporting the use of ELSD for saponins but only three for steroidal saponins [108,118,119]. Nevertheless, this detection method is not used much for routine quality control of saponin-containing herbal products.

Important progress in the detection and quantification of saponins has been achieved by combining liquid chromatography with MS detectors. In particular, HPLC with electrospray ionization mass spectrometry (HPLC-ESI-MS) or with tandem mass spectrometry (HPLC-ESI-MS/MS) have upgraded the capability to analyze the structure of saponins [108,120,122,123]. This approach still has still some limitations, however.

27.2.8 Steroidal Alkaloids

27.2.8.1 Structural Diversity and Biological Significance

Steroidal alkaloids belong to a specific but quite diverse isoprenoid alkaloid family. They are a large group characterized by the presence of an unaltered or modified steroid skeleton with nitrogen integrated either into a ring or attached just as a side chain or substituent. According to Hegenauer, these nitrogen-containing metabolites are really not true alkaloids. From a biogenetic perspective, they are just simple nitrogen derivatives of generally occurring steroidal types. At best, they can be considered as pseudoalkaloids [124]. Based on the location of the nitrogen and the skeletal arrangement, several subgroups exist (e.g., Refs. [125,126]), representing different structural types: (a) alkaloids with an unaltered C_{27} cholestane skeleton but possessing various heterocyclic rings (six skeletal types from the Solanaceae and Liliaceae families); (b) alkaloids with altered C_{27} cholestane (C-nor-D-homo-) ring systems, as well as with an 18-nor-cholestane skeleton (five types from the Liliaceae, mostly the genus *Veratrum*); (c) alkaloids with a C_{21} pregnane skeleton and with amino groups at C-3 and/or C-20, or an imino group between C-18 and C-20 (four types from the Apocynaceae and Buxaceae); and (d) alkaloids with a C_{21} skeleton and with amino groups at C-3 and/or C-20 but with one to three additional methyl groups at C-4 and/or C-14 and with 9,19 cyclopropane or 9(10)-11 *abeo* structure modification (two types from the Buxaceae). From a chemical point of view it means that nitrogen can be attached as a primary NH_2 group (free or methylated) forming simple steroidal bases, can be ring-closed to skeletal or side-chain carbons (as a secondary NH) or annelated in two rings (as a tertiary N), as, for example, in solanidine (Figure 27.1h). This often influences the chemical character and also the chromatographic properties of the compound.

Plants contain steroid alkaloids, often in glycosidic form as glycoalkaloids [127]. Moreover, all of the types a to d can contain double bonds and hydroxyls in various positions and also sugar chains, as in the case of steroidal saponins (see Section 27.2.7.1). However, the number and variety of naturally occurring glycoalkaloids is much lower than is the number of steroid glycosides [128,129]. Their distribution is also limited to a few plant families (see previous discussion). Steroidal alkaloids from the Liliaceae exceed the others in their structural, chemical, and bioactivity diversity [130]. Similarly rich in steroidal alkaloids is the Solanaceae family, which includes many important agricultural crop plants, such as potatoes, tomatoes, eggplant, and peppers [126,127]. The best known, solasodine, has been found in about 200 *Solanum* species [131]. Glycoalkaloids are generally found in all plant organs but with the highest concentrations in flowers, unripe berries, young leaves, or shoots (metabolically active parts).

Glycoalkaloids are generally toxic compounds [129,132,133]. They are also teratogens and inhibitors of acetylcholinesterase, and, like saponins, they have the ability to disrupt membranes [134].

They occur in a wide variety of plants including tomatoes and potatoes, but in the edible parts they are gradually decomposed to nitrogen-free nontoxic constituents during ripening. Moreover, levels of glycoalkaloids have been substantially reduced by breeding or by postharvest processing [135]. Throughout their long history as a human food source, potatoes and tomatoes have in general proved to be safe [136,137]. The biological activities of saponins in food are very varied and depend on both the amount and the chemical structure of the individual compounds [114,127], and in some cases also on natural microbial detoxification [138]. Various other physiological functions of solanaceous and tomato steroidal saponins are of interest for complex pharmacological studies [115].

27.2.8.2 Separation Systems and Spectroscopic Detection

For isolation of single glycoalkaloids or their aglycones, the same extraction and separation procedures are used as for steroidal saponins or sapogenins [3]. However, there is one property that can be used effectively in certain cases: Alkaloids can form water-soluble salts with acids. Crude alkaloids can then be obtained from the weakly acidic water portions of extracts by neutralization with ammonia and partitioning into an organic solvent (e.g., dichloromethane). When this procedure is applied, it is important to maintain mild conditions and consider the possible formation of artifacts.

For monitoring of steroidal glycoalkaloids, the same methods and solvent systems can be used as for steroidal saponins. TLC is still used but mainly for rapid monitoring of the hydrolysis processes employed in glycoalkaloid analysis (e.g., [139]) or of the biotransformation processes in detoxification experiments [138]. It is also occasionally used for their direct determination by HPTLC and densitometry [140]. However, in the last decades, mainly HPLC has been employed for quantification. A generally applicable method for working with small sample amounts of toxic steroidal glycoalkaloids was developed by Kubo and Fukuhara [141]. For HPLC analyses and preparations, the well-tried steroidal saponin systems [117] can be applied with modifications necessitated by the presence of alkaloid nitrogen. For rapid qualitative and quantitative analysis of steroidal alkaloids, new effective hyphenated HPLC-MS methods are increasingly applied.

27.2.9 Vertebrate-Type Steroids

27.2.9.1 Structural Diversity and Biological Significance

Progesterone and several androgens and estrogens have been detected in plant sources [142,143], but the levels are low (μg/kg) relative to the other steroids considered here, and in many cases the compounds have not been conclusively identified (e.g., estrogenic activity could derive from the presence of nonsteroidal phytoestrogens). However, immunoassay evidence indicates that vertebrate-type steroids are widespread in plants, and, in certain cases, this is supported by physicochemical identification (GC-MS, etc.). Pregnenolone and progesterone (Figure 27.1i) are intermediates in the synthesis of cardiac glycosides [12,143]. Guggulsterone ([17*E*/*Z*]pregna-4,17(20)-diene-3,16-dione; Figure 27.1j), a steroidal isomer isolated from the gum of *Commiphora mukul*, lowers LDL cholesterol levels in humans and is believed to possess cardiovascular and anti-inflammatory potential [144]. It acts as an antagonist of the farnesoid X, mineralocorticoid, glucocorticoid, and androgen nuclear receptors and an agonist of the progesterone and pregnane X receptors [145,146]. Exogenous vertebrate steroids affect plant growth and reproductive processes in various ways [143]. Vitamin D compounds (secosteroids) are present in certain plant species [147], most of which are in the Solanaceae [148,149]. These can cause calcinosis in grazing cattle, which can be a serious problem in certain parts of the world, such as Australia and South America.

27.2.9.2 Separation Systems and Spectroscopic Detection

Recent reports in this area have focused on calciferols and guggulsterone. Magalhães et al. detected ergosterol (provitamin D_2) and ergocalciferol (vitamin D_2) in hops (*Humulus lupulus*) by HPLC-DAD and HPLC-ESI-MS/MS at levels of about 2 μg/g in one hop sample [150]. They suggest that

this can be used as a marker for fungal contamination of the commercially important inflorescences, since ergosterol is essentially a fungal sterol and ergocalciferol can be derived from it by the action of UV light.

An Argentinian research group has investigated the presence of vitamin D compounds in *Nicotiana glauca* and *Solanum glaucophyllum* grown in vitro under sterile conditions and in the dark. With callus cultures of *N. glauca*, RP-HPLC eluted with MeOH–H_2O mixtures (75–90% MeOH) with monitoring at 265 nm was used to identify vitamin D_3, 25-hydroxyvitamin D_3, and 1α,25-dihydroxyvitamin D_3. Identification was confirmed by off-line MS of the HPLC peak materials [148]. A similar approach was used to demonstrate the presence of the same compounds in callus and cell cultures of *S. glaucophyllum* [149].

To assess the pharmacokinetics after guggulsterone administration, methods of RP(C_{18})-HPLC with photodiode array (PDA) detection have been developed for the quantification of guggulsterone in spiked serum samples and serum from dosed (50 mg/kg) rats. Isocratic elution with ACN–H_2O (65:35, v/v) [151] or ACN–MeOH–50 mM phosphate buffer (pH 7.2)–THF (40:25:34.5:0.5, v/v/v/v) [152], both with primary monitoring at 248 nm, was used.

Owing to the combination of the sudden large demand for guggulipid and the slow growth and poor regeneration capacity of *C. mukul*, this tree has become an endangered species. Consequently, in vitro culture techniques are being developed for the production of guggulsterone by cell cultures and micropropagation/cloning [153,154]. The levels of guggulsterone present in plant materials, cell cultures, resin, guggulipid, and pharmaceutical preparations may be determined by HPLC-PDA or HPLC-MS, using C_{18} columns eluted with gradients of ACN in H_2O [155]. Interestingly, when these methods were applied to six commercial preparations, all were found to contain significantly less guggulsterone than claimed [155].

27.2.10 Withanolides

27.2.10.1 Structural Diversity and Biological Significance

Over 200 structural analogs of withanolides have been isolated, predominantly as aglycones, although glycosides are known from some sources [156]. They occur predominantly in solanaceous plants, some of which are of great agricultural or medicinal importance. Withanolides are typically C_{28} ergostane-type steroids possessing a 22,26-δ-lactone. The side chain may be linked to the steroid nucleus 17α or 17β. There are also a large number of oxygen-containing functional groups (hydroxyls, ketones, epoxides, and cyclic ethers). Many withanolides possess a 1-oxo-group (e.g., withaferin A; Figure 27.1k). Most withanolides absorb UV light at about 220 nm in methanolic or ethanolic solutions, owing to the combined contributions of two isolated chromophores (2-en-1-one and side chain α,β-unsaturated-δ-lactone) [157]. Several of the withanolides from *Iochroma gesnerioides* antagonize ecdysteroid action in an ecdysteroid-responsive insect cell line [158] and interact with the ligand-binding domain of ecdysteroid receptors [159]. The pharmacological properties of withanolides are manifold, with suggested applications in Alzheimer's and Parkinson's diseases [160] and cancer therapy [161,162]. Many therapeutic properties have been attributed to the withanolide-containing plant *Withania somnifera*, which is used in traditional Indian systems of medicine [163].

27.2.10.2 Separation Systems and Spectroscopic Detection

Since roots of *Withania somnifera* (winter cherry/Indian ginseng) are commonly used in Ayurvedic medicine, there is an extensive literature concerning the analysis of withanolides in this species alone. Quantification of withaferin A and withanolide D from *W. somnifera* has been performed with RP-HPLC eluted at 50°C with a gradient of 35–45% MeOH–reagent alcohol in H_2O (1:1, v/v) and monitored at 230 nm [164].

RP(C_{18})-HPLC with gradient elution has been used to assess variation in levels of withaferin A in 10 commercial samples of *W. somnifera*, with the surprising result that levels were found to differ

by over 100-fold between the highest and lowest [165]. RP-HPLC contributed to the demonstration that roots of *W. somnifera* are capable of de novo biosynthesis of withanolide A [166] and to the identification of the same compound in hairy root cultures of *W. somnifera* [167].

RP(C_{18})-HPLC-PDA, eluted with a complex multi-step linear gradient with changing flow rates of MeOH (40–100%) in 0.1% aqueous acetic acid and primary monitoring at 227 nm, has been used to examine the dynamics of withaferin A and withanone accumulation and biosynthesis in leaves of *W. somnifera*. The result was that both are greatest in young leaves [168].

27.3 HYPHENATED METHODS FOR STEROID DETECTION AND QUANTIFICATION

27.3.1 Immunoassay

Immunoassays suitable for detection and quantification have been reported for only a few of the classes of phytosteroids: brassinosteroids, cardiac steroids, and ecdysteroids. The motivation for generating anti-brassinosteroid antibodies has been the desire to detect these steroids at very low concentrations in plants without the extensive work-up and derivatization procedures associated with, for example, GC-MS. Only recently has the generation of suitable antibodies been possible (e.g., [169,170]), and they have yet to find extensive application. For the cardenolides, appropriate polyclonal antibodies have existed for some time [171–173], and monoclonal antibodies [174] have been developed more recently. Immunoassays for these compounds exist in various formats: radio-immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), fluorescence polarization immunoassay (FPIA), microparticle enzyme immunoassay (MEIA), and Tina-quant [175]. They are even commercially available.

Polyclonal antibodies against the bufadienolide proscillaridin A have been used to develop an indirect ELISA assay specific for bufadienolides, which does not cross-react with cardenolides or vertebrate steroid hormones [17]. However, these antisera-recognizing cardiac steroids were produced not so much to measure the compounds in plant extracts but rather because of these compounds' effects on mammals. Equally, anti-ecdysteroid antibodies were prepared for quantification of steroid hormones in insects [176] and only later found application in analysis of plant extracts [177].

However, a major barrier to the use of immunoassays for the detection of most types of phytosteroids is the absence of suitable antibodies (poly- or mono-clonal). The difference that such antibodies can make is epitomized by research into phytoecdysteroids, where the extensive screening of simple plant extracts would not have been feasible by use of physicochemical methods. Ecdysteroid immunoassays exist in various formats (EIA, RIA, etc.), and, over the years, a number of different antisera of differing specificities have been developed. They have been used to monitor chromatographic separations of plant extracts to provide evidence for the presence of molecules chemically related to 20E, and, when combined with a bioassay to detect molecules with ecdysteroid (ant)agonist biological activities, this provides a relatively simple but very powerful approach to the dereplication of ecdysteroid-containing extracts and their component analogs (see Figure 27.3) [177]. Similar approaches would be equally useful for the other classes of phytosteroids, particularly for those for which there is no significant UV/Vis absorbance (brassinosteroids, saponins, steroidal alkaloids).

27.3.2 Bioassay

One of the most important ways of characterizing natural products is by means of their biological activity, either to identify additional members of a chemical class known to possess a particular activity or to identify new chemical classes possessing a certain biological activity. Although considerable advances have been made in the miniaturization of assays for both general and more specific activities [178], there are currently few bioassays that are fully compatible with on-line

use. However, considerable development can be expected in this area over the next few years, since coupling chromatography with on-line identification and on-line determination of biological activity is a highly desirable goal in the high-throughput screening/dereplication of plant extracts.

Suitable bioassays may be based on the use of small organisms (e.g., brine shrimp [*Artemia salina*] or the nematode *Caenorhabditis elegans*), of cells (particularly from cell cultures) for general (e.g., cytotoxicity) or specific assays, or proteins (enzymes or receptors) for specific biochemical or pharmacological assays. Bioassays are frequently performed in microtiter plates, which often permits them to be automated to achieve the initial screening of large numbers of species or subsequent bioassay-guided fractionation of active extracts. Although the key factor in the selection of the bioassay has to be the biochemical target, many other important factors must be considered: the sensitivity of the assay, the presence/absence of metabolizing enzymes, penetration to the target site, the overall robustness of the assay, the simplicity/speed of the assay, and its cost. There should also be a minimum of false negatives or false positives.

Coupling the bioassay to standardized HPLC protocols will facilitate dereplication, if they are informed by the retention times of known standards and the profiles of previously analyzed extracts [179]. An effective dereplication strategy can save much time and effort wasted in the reisolation of previously identified analogs. The pros and cons of in vitro versus in vivo assays, and the potential problems of relating activity in assays in vitro (where simplicity of interpretation is often deemed a definite advantage) to situations in vivo (i.e., the complexities of the disease state), have been comprehensively discussed recently [180].

RP-HPLC with subsequent off-line assessment of collected fractions in the rice lamina inclination bioassay was used to indicate that the liverwort *Marchantia polymorpha* contains the brassinosteroid castasterone, prior to confirmation of the identity (as the bismethaneboronate derivative) by GC-MS [181]. The need to form derivatives for GC analysis precludes the detection of biological activity following GC; thus HPLC-bioassay and GC-MS provide complementary information for these steroids without a significant UV/Vis chromophore.

An example of the use of HPLC coupled (off-line) with class-specific immunoassay and a bioassay is shown in Figure 27.3, for the separation of phytoecdysteroids from the three species in the genus *Briza* (*B. maxima*, *B. media*, and *B. minima*; family Poaceae). Most ecdysteroids absorb UV light maximally around 242 nm. Antisera have been developed for the class-specific detection of these molecules, although with differing cross-reactivities depending on the ecdysteroid analog (for example, the antiserum used in Figure 27.3 recognizes E with greater affinity than 20E). Additionally, a microplate-based bioassay, using the ecdysteroid-responsive *Drosophila melanogaster* B_{II} cell line, for the detection of molecules with ecdysteroid-like biological activity has been developed (this assay is much more sensitive to 20E than to E). In combination, these three monitoring strategies combine (i) a wavelength that is characteristic for the ecdysteroids but is not otherwise specific, (ii) an immunological test for close structural similarity to E, and (iii) a specific biological test for ecdysteroid-agonist activity [177].

All three *Briza* species are ecdysteroid-positive, but the levels differ considerably (*B. maxima* > *B. media* > *B. minima*; that is, 439:195:15 μg E eq./g with the DBL-1 antiserum). In the case of *B. maxima* (Figure 27.3.1A through 1C), the levels of ecdysteroids are so high that the ecdysteroid peaks are readily identified in the UV chromatogram (Figure 27.3.1A), and the RIA (Figure 27.3.1B) and bioassay (Figure 27.3.1C) profiles are hardly more than confirmatory. However, in the case of *B. media* (Figure 27.3.2A through 2C), the HPLC chromatogram (Figure 27.3.2A) is already more complex, and the immunoassay (Figure 27.3.2B) and bioassay (Figure 27.3.2C) data help to identify the ecdysteroid-containing fractions. With *B. minima* (Figure 27.3.3A through 3C), where the ecdysteroid levels are rather low, the much more sensitive immunological (Figure 27.3.3B) and bioassay (Figure 27.3.3C) methods are much more significant than the UV trace in identifying the ecdysteroids present.

Certain cucurbitacins and withanolides can be detected in the bioassay for ecdysteroids mentioned previously [182], owing to their activity as ecdysteroid antagonists [34,158].

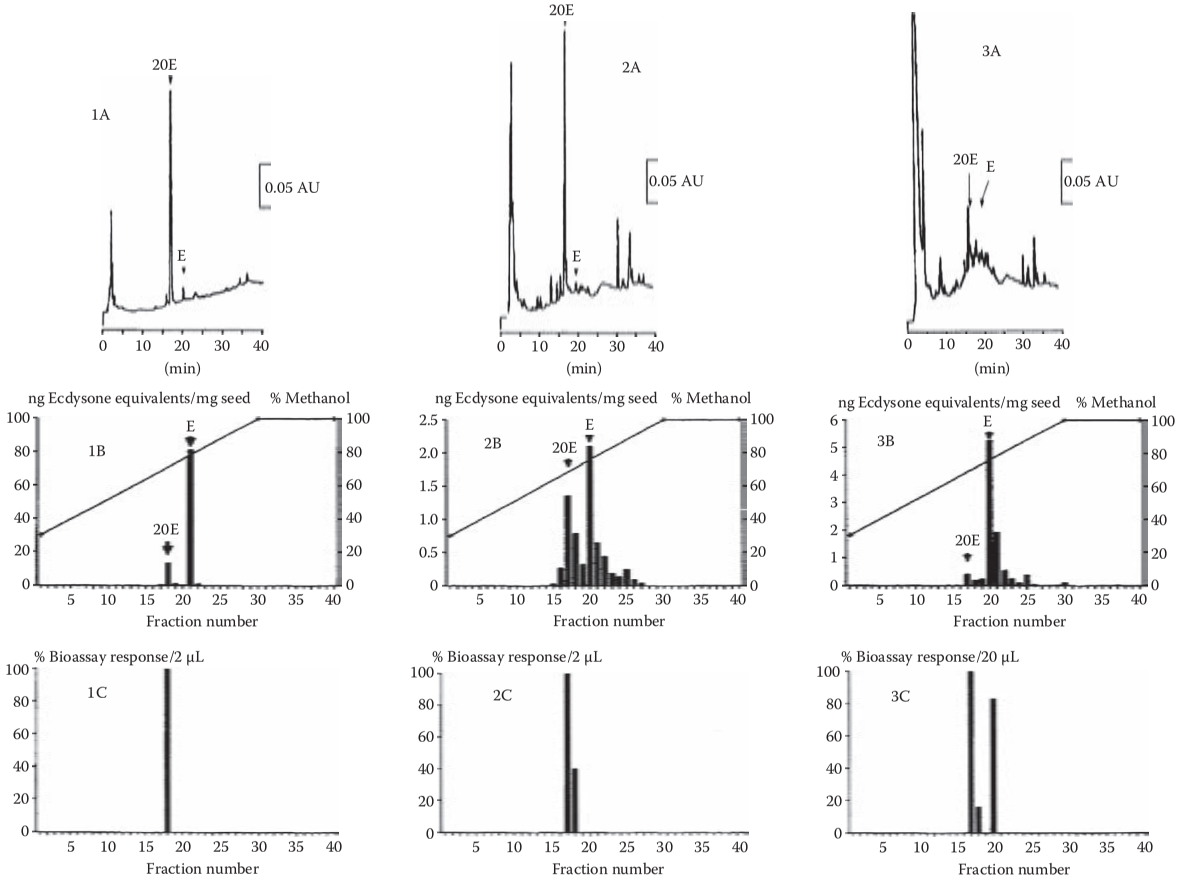

FIGURE 27.3 RP-HPLC/UV/RIA/bioassay of extracts of seeds of *Briza maxima* (1A, B & C), *B. media* (2A, B & C) and *B. minima* (3A, B & C). Equivalent weights (25 mg) of seeds of each species were extracted with methanol (3x) and, after partitioning against hexane, aliquots were separated on an analytical Spherisorb ODS-2 column eluted with a gradient of MeOH in H_2O (shown in the B-panels) at 1mL/min and monitored at 242 nm (A-panels). Fractions of 1mL were collected and aliquots were assessed by RIA ('Black' antiserum; B-panels) and in the agonist version of the B_{II} bioassay (C-panels). E: ecdysone; 20E: 20-hydroxyecdysone. (Redrawn from Dinan, l., *Eur. J. Entomol.*, 92, 271–283, 1995.)

27.3.3 Direct Detection and Postcolumn Derivatization

Depending on the steroids' chemical structure and concentration in biological samples, different techniques are used. UV detection is by far the most common procedure, provided that the steroids possess a suitable chromophore (e.g., a conjugated diene, an α,β-unsaturated ketone). DAD provides more information, as a wide range of wavelengths is monitored throughout the chromatographic separation, and the absorbance spectrum of each eluted peak is obtained, which is also important to assess its purity. When a single C-C double bond is present (e.g., in many sterols), compounds absorb only at around 205 nm, which is not at all specific, creating several drawbacks such as reduced sensitivity and accuracy.

When no unsaturation is present (e.g., stanols, saponins), UV detection cannot be used, and in such cases, the only available detector was initially the differential refractometer, which allows only isocratic chromatographic systems to be used. Fortunately, a new type of universal mass-specific detector was recently introduced, the ELSD, which accommodates almost any type of solvent and has become very popular for the analysis of natural crude product mixtures. An example of combined use of UV detection and ELSD is given in Figure 27.4. ELSD has been used with various classes of plant steroids, such as ecdysteroids [183], phytosterols [184], and steroidal saponins [108,118,119].

Fluorescence detection, when appropriate, provides greater sensitivity than UV/Vis spectrophotometry. Accordingly, it may be of interest to convert steroids into fluorescent derivatives, which may be done either before or after HPLC analysis. Precolumn derivatization presents several drawbacks: The reaction may be incomplete or lead to a mixture of several derivatives from a single compound. This approach has been successfully used for ecdysteroids using anthroyl derivatives [62] and for ginsenosides (saponins) using ozonolysis followed by reaction with fluoroenylmethyl oxycarbonyl chloride (FMOC)-hydrazine [185]. Postcolumn derivatization can be performed on-line or off-line. In-line procedures require a specific reactor, but this is, of course, the more convenient mode. Many steroids give rise to fluorescent derivatives upon treatment with concentrated sulfuric acid [186], and this procedure has been used off-line for ecdysteroids [186] and in-line for cardenolides [187].

Pulsed amperometric detection (PAD) represents another alternative detection technique, especially suitable for the detection of polar aliphatic compounds like carbohydrates. This particular electrochemical detection method uses a three-step waveform voltage applied to the detection electrode and measures the electrical current generated when analytes pass into the detector cell (e.g., [188]). In the case of cardiac glycosides, it provides a sensitive method, allowing the detection of low nanogram amounts, whereas UV detection at 220 nm is not particularly specific or sensitive [189].

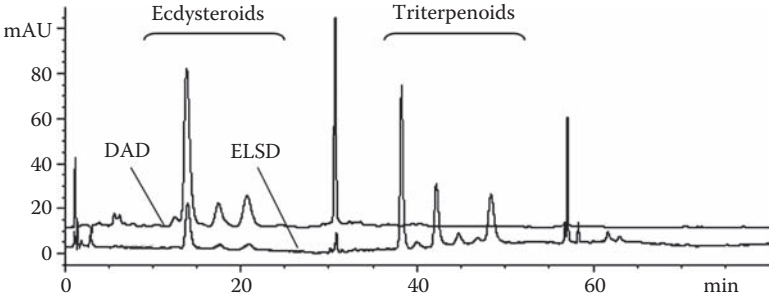

FIGURE 27.4 HPLC-diode-array detection–evaporative light-scattering detection chromatogram of an extract of Radix *Achyranthes bidentatae* containing both ecdysteroids and triterpenoid saponins. Reversed-phase HPLC used a discontinuous gradient of acetonitrile in water containing 5% (v/v) propan-2-ol and 0.08% (v/v) formic acid. (Redrawn from Li, J., Li, H.-J., Li, P., and Qi, H.,, *Biomed. Chromatogr.*, 21, 823–828, 2007.)

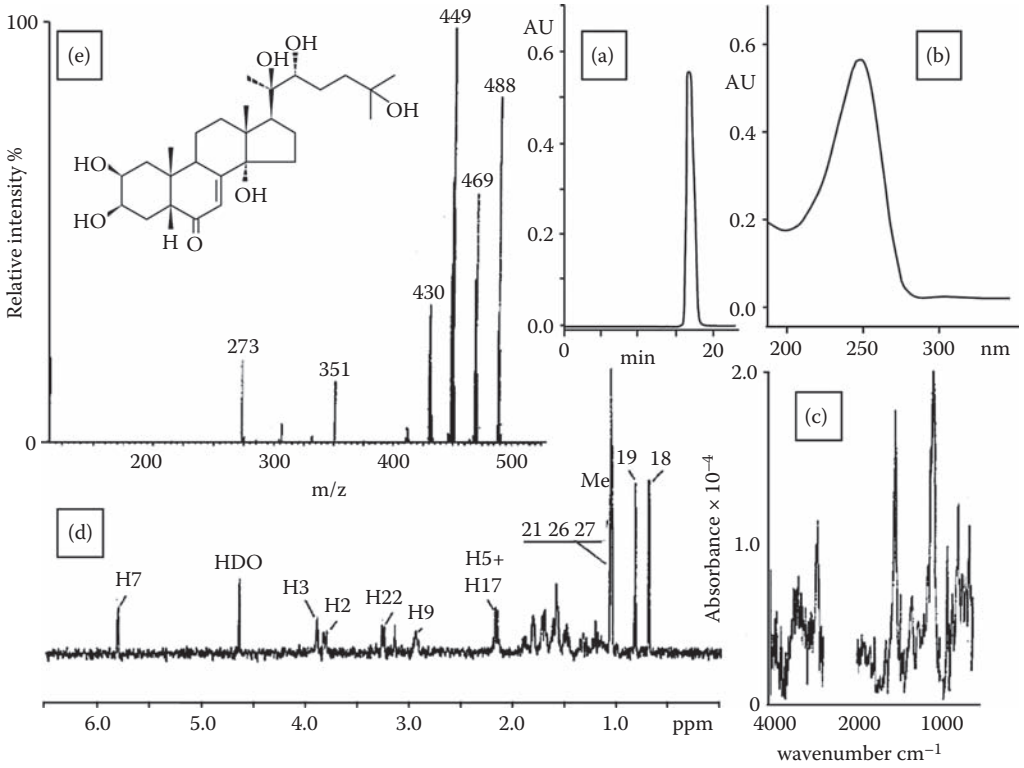

FIGURE 27.5 Chromatographic and spectroscopic data obtained with 200 µg 20-hydroxyecdysone (20E) using multihyphenated HPLC: (A) HPLC-UV (254 nm) chromatogram and (B) UV spectrum; (C) Fourier transform infrared detector; (D) ^{1}H-nuclear magnetic resonance (NMR); and (E) mass spectra obtained from the 20E peak eluting at 17 min. (Redrawn from Louden, D., Handley, A., Lafont, R., Taylor, S., Sinclair, I., Lenz, E., Orton, T., and Wilson, I.D., *Anal. Chem.*, 74, 288–294, 2002.)

27.3.4 MS

For a general review of this topic, see [190]. Chemical screening for pharmaceutical purposes requires equipment that can provide maximal information within a short time, and for that purpose HPLC and mass spectrometers are interfaced, a technique that has undergone spectacular developments over the last 10 years and now provides a particularly efficient tool [191]. Interfacing MS with HPLC is not an easy task, and although the first attempts were performed about 30 years ago (see the review by Niessen [192]), the instruments became more functional only with the development of thermospray (TSP; for ecdysteroids, see, e.g., [193]) and then atmospheric pressure ionization (API) interfaces. The latter allows thermolabile molecules to be analyzed. These techniques use a combination of heat and high voltage to produce ions, and two types have been developed: ESI (e.g., for ecdysteroids [194–197]) and APCI (e.g., [197]). These soft ionization methods require RP-HPLC in which some acid (acetic or formic acid) is added to the mobile phase; they produce first-order ions, that is, a mixture of $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ ions in the positive mode or $[M-H]^-$ ions in the negative mode.

Mass analyzers are essentially multiple-stage machines (MS/MS), such as triple quadrupole (QqQ) or quadrupole-time-of-flight (Q-TOF) spectrometers, which allow the analysis of secondary ions arising from any selected first-order ion and therefore represent a very specific detection procedure. More sophisticated machines (ion trap coupled to an orbitrap) allow high-resolution mass spectrometry (HRMS) determinations to be performed (e.g., [198]), which provide useful

information about the elemental composition of each ion and thus allow an improved certainty of identification when complex samples are analyzed. The different HPLC-MS techniques have been successfully used for almost every class of phytosteroids, for example, bufadienolides [199], cucurbitacins [200–202], phytosterols [83,84,87–90,92,93], saponins [108,120,122,130,203–205], steroidal alkaloids [206,207], and withanolides [208–211].

However, although they provide a lot of structural information, even the more advanced HPLC-MS methods are not sufficient in the case of complex molecules like saponins, since they cannot resolve the position of attachment of sugars, or their isomeric character; that is, they cannot provide total structural information. Thus, only NMR analysis of isolated compounds, or NMR coupled with HPLC (LC-NMR), together with complementary rapid LC-UV-MS screening, generates adequate structural information for total and unambiguous structural identification [212].

27.3.5 Multiple On-line Detection Methods

It may appear beneficial to take advantage of the numerous spectroscopic techniques available and couple them sequentially or in parallel to HPLC as multiple-hyphenation systems (e.g., Figure 27.5). This could replace the classical (and time-consuming) isolation of individual compounds from complex matrices, followed by their off-line spectroscopic (MS, NMR) characterization [212,213]. Only NMR can provide the unambiguous structural elucidation of every compound, especially when no reference molecules are available. For this reason, placing all the required identification techniques in a single multiple-hyphenated apparatus appears very attractive, although it is evident that this raises several technical problems associated with the different sensitivities of the different techniques and/or their specific solvent requirements. As a consequence, the resultant method has to be a compromise that does not allow the optimal use of individual techniques, but it has been demonstrated to be operational in several instances [213]. Fourier-transform infrared detectors can be interfaced with HPLC [64,214,215] and thus provide useful structural information with regard to the presence of specific functional groups. HPLC-infrared detection has limited sensitivity compared with UV. It can be operated in two different ways, using either a flow cell or, better, a solvent elimination procedure [215].

The most powerful technique for full structural identification is NMR, which can also be interfaced with HPLC in the RP mode, provided that deuterated solvents (D_2O, CD_3OD, CD_3CN) are used (see Refs. [64,216,217]). The use of superheated D_2O as the HPLC mobile phase even avoids the need for organic modifiers [214].

27.3.6 Designing a General "Routine" System

When applicable, on-line detection procedures are preferable, as they are less time-consuming, but they require sophisticated equipment. Among them, HPLC-DAD-MS appears the most suitable system [218]; although unable to fully establish the identity of compounds, it allows their characterization based on three different criteria: retention time, (UV) absorbance spectrum, and mass spectrum (including elemental composition based on high-resolution mass spectra, when available). This procedure is currently used as a "routine" analytical method [53,195–197,219]. Of course, an ELSD can be added to or can replace the DAD to obtain a more universal detection system [183,220].

27.4 CONCLUSIONS AND PROSPECTS

Phytosteroids are a very diverse group of molecules that can be divided into several classes, each consisting of many known analogs and probably many as yet undetermined analogs. In addition to their hormonal and chemical ecological roles within plants, the different classes of phytosteroids show many interesting pharmacological properties, giving them considerable biotechnological

potential. HPLC is extensively applied to most aspects of current research on all classes of phytosteroids, but there have been few systematic studies on the chromatographic behavior of a significant number of analogs from within individual classes of phytosteroids. The ecdysteroids form a notable exception, as a large number of their analogs have been studied in several HPLC systems, as in [53].

For other classes of phytosteroids, chromatographic solutions are sought on a "needs must" basis. While understandable, this is unfortunate, since we do not possess the body of knowledge that would assist the identification and dereplication of these classes. Emphasis is currently on the development of coupled techniques, which permit more unambiguous identification but at higher cost and technical complexity. New developments in detectors are aimed at more sensitive methods of universal detection and the use of biosensors to detect compounds with particular biological activities.

In view of the complexity of the profiles of each of the classes of phytosteroids typically found in plant species and the growing realization of the potential pharmacological significance of these molecules, one can expect, on the one hand, that two-dimensional HPLC [221,222] will be applied to complex mixtures to better resolve the many analogs and, on the other hand, that on-line coupled techniques [192,223], and even combinations of these [224], will be more extensively used for identification and dereplication of analogs.

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28 HPLC of Iridoids

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CONTENTS

28.1 INTRODUCTION

The iridoids are a large group of naturally occurring monoterpenoids containing a dimethylcyclopenta[*c*]pyran (iridane, or 2-oxabicyclo[4.3.0]nonane) skeleton (Figure 28.1). The vast majority of iridoids are found in higher plants. Their ecological functions, physiological activity and pharmacology, and significance as chemotaxonomic markers have attracted considerable interest. More than 1,500 distinct structures have been reported. Several review articles list most of the isolated iridoids along with their physical and spectral data and original sources [1–6].

28.2 STRUCTURE, DISTRIBUTION, AND BIOLOGICAL ACTIVITY OF IRIDOIDS

28.2.1 Structural Groups

Iridoids occur mainly as 1-*O*-glucosides, but a small number of nonglycosidic iridoids in the forms of ester, acetal, and hemiacetal forms are also known [1–6]. Stable iridoid aglycones are, for example, nepetalactone and *Teucrium* lactones. They are volatile and therefore occasionally reported as components of essential oils [7]. Iridoids are biosynthesized in plants from geranylpyrophosphate via hydroxynerol and then iridodial (Figure 28.2). The latter exists in equilibrium with its hemiacetal form. A cleavage of the cyclopentane ring at the bond between carbons 7 and 8 yields the secoiridoids. The great variations in the iridoid structures are attained by (1) direct modification of the iridane skeleton, such as loss of carbons at the 4 and/or 8 position, (2) oxidation of the skeleton to aldehyde or acid and epoxidation with specific stereostructures,

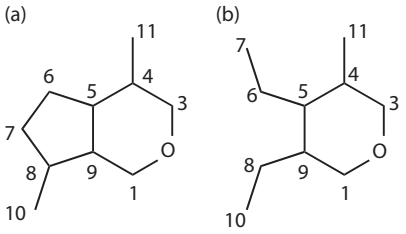

FIGURE 28.1 Chemical structure of iridoids: (a) iridane, (b) secoiridoid.

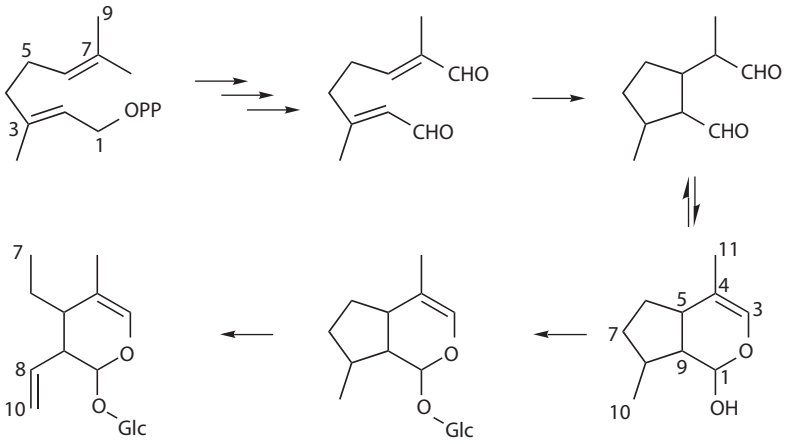

FIGURE 28.2 Biosynthetic pathway to iridoids and secoiridoids.

(3) dehydration and formation of double bonds, (4) further substitution of the functional groups by glycosidic moieties, aliphatic and aromatic organic acids, or inorganic acids (e.g., sulfate), (5) conjugation with other classes of compounds including amino acids, phenylpropanoid derivatives such as coumarins and lignans, monoterpenoids, and alkaloids, and (6) oligomerization of iridoids and secoiridoids (Figure 28.3) [1–6].

28.2.2 Distribution in Plants and Chemosystematics

Except for some compounds found in insects and marine organisms, most iridoids are plant secondary metabolites. They have been found only in the clade Asterids, according to the APG classification of the flowering plants [8]. Iridoids are the main secondary metabolites in the orders Cornales and Ericales as well as in clade Euasterids I, which comprises the orders Gerryales, Gentianales, Lamiales and Solanales. They have also been found in low concentrations in some representatives of the closely related clade Euasterids II. It has been assumed that the genes for iridoid biosynthesis are present in both clades [9]. However, in the Euasterids II most of the available isoprene precursors are involved in the synthesis of other terpenoids that have a larger potential as protective substances than iridoids, such as sesquiterpenoids in the Asteraceae [10]. Iridoids have not been found in any other groups of plants including monocotyledons, gymnosperms, and pteridophytes, or fungi.

Chemotaxonomy, or micromolecular systematics, uses low-molecular-weight plant metabolites (secondary metabolites) as taxonomic characteristics to assist the reconstruction of the phylogenetic relationships of plants. Hegnauer first recognized the significance of iridoids in the taxonomy of the sympetalous families [11]. Jensen et al. and Dahlgren have used the patterns of occurrence of iridoids as important characteristics in the classification of the dicotyledons [12,13]. The degree

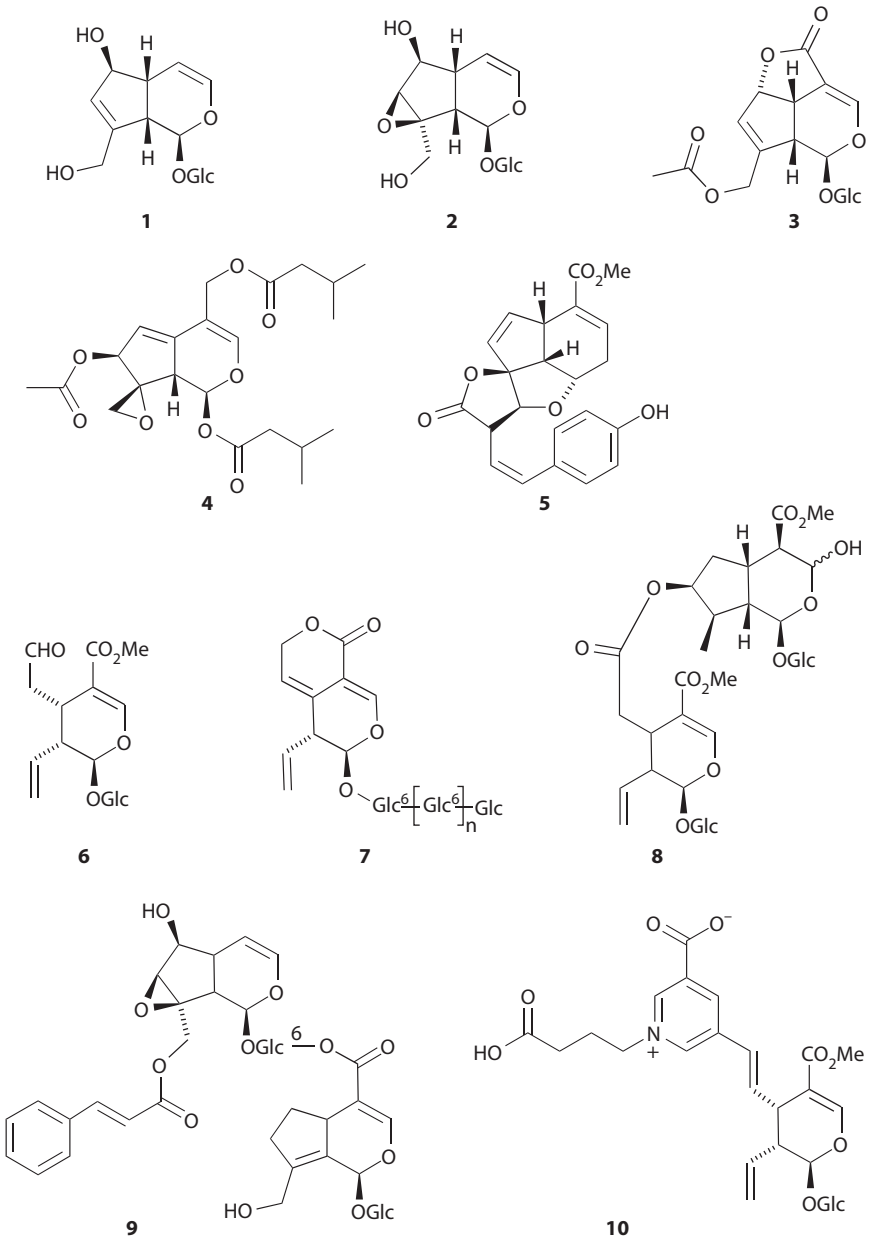

FIGURE 28.3 Structural variations of iridoids and secoiridoids. 1, aucubin; 2, catalpol; 3, asperuloside; 4, isovaltrate; 5, prismatomerin; 6, secologanin; 7, scabrans ($n = 1 \sim 3$); 8, loasafolioside; 9, wulfenoside; 10, lonijaposide A.

of oxidation and skeletal specialization, as well as biosynthetic pathways, are also important for chemosystematic purposes [14–16].

Iridoids have been considered one of the most useful groups of secondary metabolites for classifying species at lower taxonomic ranks [17,18]. The relationships between species and families implied by the iridoid characteristics are remarkably congruent with those derived independently from analyses of DNA sequence data [9]. Iridoid markers have been used in the chemosystematic studies of Verbenaceae [14], Acanthaceae and its allies [15], Plantaginaceae [19–21, and the references therein], Oleaceae [22], Bignoniaceae [23], Loasaceae [24,25], and Valerianaceae and its allies [26].

28.2.3 Ecological Functions and Pharmacology of Iridoids

A series of studies has shown the ecological role of iridoids in plants responding to biotic and abiotic stresses. They are also considered a key factor in some plant–plant and plant–herbivore relationships [27]. Thus, aucubin (Figure 28.3, **1**) and catalpol (Figure 28.3, **2**) in *Plantago lanceolata* are shown to be involved in feeding avoidance by polyphagous insects such as *Spodoptera exigua* [28]. Fuchs and Bowers observed significant changes in the composition and concentration of iridoids in plants injured by feeding insects [29]. Konno et al. showed that when the leaf tissue of privet tree (*Ligustrum obtusifolium*) is injured by herbivores, plant's enzyme systems convert the main iridoid, oleuropein, into a strong protein denaturant, the action of which significantly decreases the nutritive value of dietary protein [30]. However, the high concentration of free glycine found in the digestive juice of the larva of *Brahmaea wallichii* works as a neutralizer against the protein-denaturing activity of oleuropein and allows the larvae to feed on the privet tree. Some insect herbivores evolved even further to exploit the ingested iridoids for their own defense [31]. In some cases, iridoid glycosides in plants serve as feeding attractants for insect larvae [32] or are oviposition stimulants [33]. Aucubin and catalpol, and the secoiridoid oleuropein, are precursors to potent antifungal agents that restrain the growth of fungal pathogens on plants [28,34]. Pardo et al. have demonstrated that the iridoids harpagoside, lateroside, and aucubin were released from the roots of *Verbascum thapsus* into the surrounding environment (rhizodeposition) to suppress germination of proximate barley seeds and thus provide competitive strength for the species [35].

Iridoid-containing plants have been used in pharmacology in various formulations. Iridoids of different classes are components of cough medicines, bitter tonics, antiseptics and various healing remedies, sedatives, and hypotensives. A wide range of biological and pharmacological activities are known [6,36,37]. Valepotriates (e.g., Figure 28.3, **4**) from the Valerianaceae have a distinctive sedative effect, while Gentianaceae and Menyanthaceae are sources of bitter tonics. The anti-inflammatory action of harpagoside, the major constituent of *Harpagophytum*, has been used to treat rheumatoid and osteoarthritis. PicroLive, a standardized iridoid fraction from rhizome of *Picrorhiza kurroa*, has a strong hepatoprotective activity and has been used as such. Aucubin and catalpol, found in many medicinal plants, exhibit anti-inflammatory, antimicrobial, and antiviral activities. Epoxy- and methoxy-gaertnerosides, antiamoebic iridoids from *Morinda morindoides*, displayed strong mutual synergistic effects when re-mixed with the crude extract from which the compounds were isolated [38]. Several articles review the medicinal properties of iridoid-containing plants and their constituents, for example, the iridoids in *Buddleja* [39], *Cornus* [40], *Eucommia* [41], and *Plantago* [42].

28.3 EXTRACTION AND ISOLATION OF IRIDOIDS

Iridoid and secoiridoid glycosides are generally considered unstable compounds that may undergo degradation by a trace of acid or prolonged exposure to temperatures higher than 60°C [43]. Therefore, mild extraction techniques and rapid chromatographic methods are used for their purification. A variety of techniques for handling iridoid-containing plants can be found in several review articles [43–46].

28.3.1 Extraction and Preliminary Purification

To prevent iridoid degradation by the plant's own enzyme system, the plant material is best kept frozen, lyophilized, or dried (below 40°C) immediately after collection ([43] and references therein). Total iridoid extracts are usually obtained by maceration with either water, ethanol, methanol (MeOH), or acetone, or mixtures of these, at room temperature [43,45]. Extraction in an ultrasonic bath has been used to accelerate the diffusion of the compounds, with extraction time ranging from 10 to 60 min [47–50]. Suomi et al. compared the efficiency of pressurized liquid extraction and

maceration with water and various organic solvents for the isolation of aucubin and catalpol from *Veronica longifolia* [51].

The crude plant extract is usually concentrated *in vacuo*, and nonpolar contaminants (chlorophyll, lipids, etc.) are removed by extraction with nonpolar solvents such as petroleum ether, diethyl ether, or dichloroethane. Some phenolics remain in the aqueous layer and may interfere with the subsequent isolation of iridoids. These are often removed by liquid–liquid extraction with solvents of intermediate polarity (ethyl acetate, *n*-butanol) against water [43,45].

Multiple steps of chromatographic purification are usually required to separate the individual iridoids. The initial chromatographic separation involves methods with high-capacity and less costly stationary phases such as neutral alumina, activated charcoal, or porous polystyrene-divinylbenzene (PS–DVB) polymers (e.g., Amberlite XAD; Diaion HP-20, HP-21). The obtained fractions, enriched with iridoid glycosides, are further subjected to various separation techniques. Column chromatography (CC) over silica gel is usually carried out with chloroform–MeOH–H_2O mixtures with increasing polarity; the presence of water ensures an element of partition chromatography as well as improving the solubility. Polyamide and Sephadex LH-20 are used with MeOH–H_2O mixtures as eluents when a crude iridoid extract contains a large amount of phenolics. Common techniques for isolation of iridoids are low pressure liquid chromatography (LPLC, up to 10 bar) and medium pressure liquid chromatography (MPLC, up to 20 bar), with C_8 and C_{18} stationary phases, eluted with MeOH–H_2O mixtures as mobile phases [43,52]. The application of countercurrent techniques such as rotation locular countercurrent chromatography (RLCCC) and droplet countercurrent chromatography (DCCC) is summarized by Junior [44]. In these methods the solid stationary phase is replaced by immiscible liquid medium, and the injected samples are recovered quantitatively. Other chromatographic techniques with relatively limited application in the isolation of iridoids are preparative thin-layer chromatography (PTLC), centrifugal thin-layer chromatography (CTLC), overpressure layer chromatography (OPLC), and vacuum liquid chromatography (VLC) [43].

28.3.2 Preparative HPLC: Mobile and Stationary Phases

Preparative HPLC is used as a purification step in the isolation of iridoids, usually for fractions already enriched in iridoids by other chromatographic techniques. The average particle size of preparative-HPLC stationary phases is between 5 and 10 μm and the column diameter is usually in the range of 10 to 20 mm. A 57 mm-diameter column was used for isolation of aucubin and its derivatives [53]. Depending on the purpose of the isolation, analytical-sized columns could also be used, if the amount required for subsequent analyses and/or bioactivity testing is small, or the column capacity may be raised [54,55]. Thus, an analytical C_{18} column of 4.6 mm diameter (×150 mm length) was used for final purification of eight nonglycosidic and modified iridoids from the leaves of *Catalpa ovata* (MeOH–H_2O, 40:60, 1.5 mL/min, operated at 40°C, detection with UV at 314 nm), yielding from 5.7 to 27.5 mg of each compound [56]. Packing materials used in preparative HPLC are usually identical to those used in analytical HPLC, which facilitates a direct transition of the optimized methods to preparative scale. Semipreparative HPLC is usually applied for separation of 1–100 mg of sample, depending on the sample's complexity and the required resolution [46].

Some examples of application of preparative and semipreparative HPLC in the isolation of iridoids are given in Table 28.1. Most of the reported methods use octadecylsilyl phases in isocratic [57–59] or gradient conditions [60–62]. In most cases MeOH–H_2O or MeCN (acetonitrile)–H_2O binary mixtures are employed as solvent systems. Seger et al. used a more complex multistage gradient for isolation of harpagide *p*-coumaroyl esters on a Zorbax SB-C_{18} column (4.6 × 150 mm; particle diam. 3.5 μm) [55]. Addition of acid to the mobile phase is usually avoided to prevent degradation during the recovery of the separated compounds. Some examples are preparative HPLC of the iridoids in *Paederia scandens* [58], *Syringa dilatata* [62], *Patrinia scabra* [63], and *Vitex altissima* [64]. Nevertheless, small amounts of acid were added in some cases to improve separation

TABLE 28.1
Examples of Preparative and Semipreparative HPLC of Iridoids

Column[1]	Mobile Phase[2]	Flow Rate[3]	Detection[4]	Compounds/Plants	Ref.
Reversed phase — C_{18}					
HS Hyperprep 100 BDS C_{18}[a], 10 × 250 (8 µm)	MeCN–H_2O (20:80 → 35:65)	2	UV	Syringopicroside; *Syringa dilatata*	[62]
Prevail[a], 10 × 250 (5 µm)	MeCN–H_2O–HOAc (11:89:0.5)	—[6]	UV	Lonijaposide A; *Lonicera japonica*	[59]
Luna C_{18}[b], 21.2 × 250	MeCN–H_2O (25:75, 15:85)	20	UV	Gardoside mono- and diesters; *Vitex altissima*	[64]
Zorbax SB-C_{18}[c], 4.6 × 150 (3.5 µm); 50°C	MeCN–H_2O (both with 0.1% HOAc) (2:98 →→ 98:2)	1	UV	Harpagide *p*-coumaroyl esters; *Harpagophytum* spp.	[55]
LiChrosorb RP-18[d], 16 × 250 (7 µm)	MeCN–H_2O (15:85 → 50:50)	—	UV	Loasafolioside; *Loasa acerifolia*	[61]
Spherisorb S10 ODS2[e], 20 × 250 (10 µm)	MeCN–MeOH–H_2O (15:7:78)	5	UV	8-*O*-Acetylmioporoside; *Ajuga salicifolia*	[57]
Nucleosil[f], 4 × 250 (7 µm)	MeOH–H_2O, with 0.05% TFA (45:55)	1	UV	Blumeoside C; *Fagraea blumei*	[54]
PrepPak C_{18}[e], 25 × 100 (6 µm)[5]	MeOH–H_2O–TFA (20:80:0.1 → 80:20:0.1)	5	UV	Ipolamiide; *Stachytarpheta cayennensis*	[60]
Betasil C_{18}[g], 10 × 250 (5 µm)	MeOH–H_2O (50:50)	2	—	Paederoside B; *Paederia scandens*	[58]
Reversed phase — Phenyl					
C-Phenyl[b], 21.2 × 250	MeOH–H_2O–HOAc (23:77:0.5)	10	UV	Nonglycosidic iridoids; *Verbena littoralis*	[72]
Amide/amino phases					
TSKgel Amide-80[h], 7.8 × 300; 40°C	MeCN–H_2O (75:25)	1.5	RI	Scabrans G3, G4 and G5; *Gentiana scabra*	[65]
YMC-NH_2[i]	MeCN–H_2O (90:10)	1	RI	Littoralisone; *Verbena littoralis*	[66]
Normal phase					
Supelcosil SPLC-Si[j], 10 × 250	Cyclohexane–EtOAc (90:10)	—	RI	Nepetaparnone; *Nepeta parnassica*	[67]
Chiraspher[d], 10 × 250	Hexane–MeOH–iPrOH (77:13.1:9.9)	—	UV	7-Feruloylloganin; *Lonicera insularis*	[71]

[1] Brand, diameter and length (mm), particle diameter, and operating temperature where reported. Column manufacturers: a, Alltech; b, Phenomenex; c, Agilent Technologies; d, Merck; e, Waters Associates; f, Macherey-Nagel; g, Keystone; h, Tosoh; i, YMC; j, Supelco.

[2] Mobile-phase components in volume ratio. An arrow (→) denotes a linear gradient; double arrows (→→) denote sectioned (discontinuous, multistep) gradients.

[3] In mL/min.

[4] UV, ultraviolet absorption, including photodiode-array detection; RI, refractive index.

[5] Radial compression column, operated at 600–700 psi.

[6] Not reported.

of acylated compounds, such as the iridoid esters from *Harpagophytum procumbens* [55]. Song et al. have isolated pyridinium alkaloid-coupled secoiridoid lonijaposide A (Figure 28.3, **10**) from *Lonicera japonica* using a mobile phase of MeCN–H_2O (11:89) containing 0.5% acetic acid [59].

Columns with amide- and amino-embedded phases, suitable for more polar compounds, have occasionally been used in preparative HPLC of iridoids. Secoiridoid glycosides with more than three glucose units (scabrans, Figure 28.3, **7**) were purified on an amide phase with MeCN–H_2O (3:1) in isocratic mode [65]. Similarly, Li et al. used a YMC-NH_2 column to isolate the heptacyclic iridoid littoralisone from *Verbena littoralis*, with a MeCN–H_2O mixture (9:1) in isocratic mode [66].

Normal-phase columns, suitable for nonpolar iridoids, have also been used. Two nonglycosidic iridoids (nepetalactone dimers) were isolated by normal-phase HPLC using cyclohexane–ethyl acetate as an eluent [67]. In several cases the compounds were separated first on reversed-phase HPLC followed by normal-phase HPLC as a final purification step [68–70]. A polyacrylamide-coated silica column (ChiraSpher; Merck) was used in an analogous manner to normal phase for the isolation of 7-feruloylloganin from *Lonicera insularis*, with the solvent system hexane–MeOH–isopropanol (77:13.1:9.9) [71].

UV and refractive index (RI) detectors are most often employed in preparative and semipreparative HPLC of iridoids. These detection techniques are nondestructive and permit compound recovery after the chromatographic separation. UV detection for iridoids with carbonyl group (aldehyde, carboxylic acid, ester) at C-4 was carried out at wavelength range 240–270 nm [60,62,72,73]. Detection at 205–215 nm was required for non-UV-absorbing iridoid glycosides [61,74]. The RI detector, in conjunction with isocratic elution programs, has been used to separate patrinoside [63], littoralisone [66], and nepetaparnone [67].

28.4 HPLC ANALYSIS OF IRIDOIDS

HPLC is a powerful tool for the detection, quantitation, and estimation of purity of natural compounds, including iridoids. The difference between the analytical and preparative methodologies reflects the purpose of the chromatographic separation. Preparative and semipreparative HPLC are purification techniques that aim at the isolation and recovery of compounds, while analytical HPLC is used for qualitative and quantitative analyses.

A modern HPLC system comprises several distinct components: a pump to deliver solvents of defined composition at a high pressure (up to 600 bar, ~9000 psi), autosampler, column(s), and one or more detectors. The hardware is usually controlled by a computer system, which allows a high degree of precision and reproducibility. The great resolving power and high-speed separation of HPLC are the main advantages that make this technique so popular in studies of the relatively unstable iridoids, which are usually present in very complex plant matrices.

28.4.1 Stationary Phases

A great variety of columns with different chemistry, particle size, and dimensions are available on the market now. Older type A silica-based particles with irregular size and shape have been replaced entirely by spherical, low-iron-content synthetic silica particles (type B). Octadecylsilano-modified silica particles in reversed-phase modes (ODS, C_{18}) are the most popular column chemistry in HPLC of iridoids (Table 28.2). Amino/amide-embedded and phenyl-bonded reversed-phase columns have been used for analysis of the cinnamoyl and benzoyl esters of catalpol [75] and the nonglycosidic iridoid, prismatomerin (Figure 28.3, **5**) [76]. Ion-exchange chromatography was used by Marak et al. for the analysis of aucubin and catalpol, highly polar iridoid glucosides with low retention on reversed-phase columns [77].

Monolithic columns, where the stationary phase is based on a solid porous structure of high-purity silica, have also been used in iridoid analysis. They provide high-performance and high-speed

TABLE 28.2
Examples of Analytical HPLC of Iridoids

Column[1]	Mobile Phase[2]	Flow Rate[3]	Detection[4]	Compounds and Plants	Ref.
Reversed phase — C_{18}					
Alltima C_{18}[a], 4.6 × 250 (5 µm); ambient temp.	MeCN–H_2O (0:100 →→ 90:10)	1	UV	Asperuloside, 6-*O*-*p*-coumaroylscandoside; *Oldenlandia* spp.	[87]
µBondapak C_{18}[b], 3.9 × 300 (10 µm)	MeOH–H_2O (57:43)	1	UV	Harpagoside, 8-*O*-*p*-coumaroylharpagide; *Harpagophytum procumbens*, *H. zeyheri*	[98]
Chromolith RP-18e[c], 4.6 × 100, two columns in series; 30°C	MeCN–H_2O (pH 2.0, H_3PO_4) (1:99 → 50:50)	5.0	UV	Harpagoside, 8-*O*-*p*-coumaroylharpagide; *Harpagophytum procumbens*	[78,118]
Hypersil[d], 4.6 × 200 (5 µm)	MeCN–H_2O–H_3PO_4 (20:80:0.05 → 80:20:0.05)	1	UV	Valtrate, isovaltrate; *Valeriana officinalis*	[108]
Hypersil[d], 4 × 250 (5 µm)	MeCN–aq. 3% HOAc (5:95 →→ 90:10)	0.8	UV	Gentiopicroside, sweroside, swertiamarin; *Centaurium erythraea*	[86]
LiChroCART 5RP_{18}[c], 4 × 150	MeCN–H_2O (75:25); MeCN–H_2O (77.5:22.5)	1	UV	Mussaenosidic acid 2′-esters; *Avicennia* spp.	[107]
LiChrospher 100RP[c], 4 × 250 (5 µm); ambient temp.	MeCN–H_2O (pH 4 with H_3PO_4) (0:100 →→ 100:0)	1.2	UV	Aucubin, catalpol, harpagoside; *Scrophularia nodosa*	[119]
Nucleosil[e], 4 × 250 (5 µm)	MeOH–H_2O (70:30)	1.2	UV	Isovaltrate, didrovaltrate; *Valeriana officinalis*	[81]
Necleosil RP-18[e], 4 × 250 (7 µm)	MeCN–aq. 0.05% TFA (5:95 → 65:35)	—[5]	UV	6‴′-*O*-Glucosyltrifloroside; *Gentiana linearis*	[103]
Nucleosil C18[e], 4.6 × 125 (5 µm); 40°C	MeCN–H_2O (3:97)	—	UV	Antirrhinoside, antirrhide, 5-glucosylantirrhinoside, linarioside; *Antirrhinum majus*	[49]
Spherisorb ODS2[e], 4 × 125 (5 µm)	MeOH–H_2O	0.8	UV	Aucubin, catalpol; *Paulownia tomentosa*	[120]
Symmetry[b], 3 × 250 (3 µm)	MeCN–H_2O (10:90 → 50:50)	1.0	UV	Verbenalin, hastatoside; *Verbena officinalis*	[110]
Ultracarb C20[f], 4.6 × 150 (5 µm); ambient temp.	MeOH–H_2O (30:70)	1	UV	Swertiamarin, gentiopicroside, sweroside; *Gentiana lutea*	[79,80]
Zorbax SB-C_{18}[g], 4.6 × 250 (5 µm); 30°C	MeCN–aq. 1% H_3PO_4 (15.4:84.6 →→ 100:0)	1	UV	Secologanoside deriv.; *Olea europaea*	[121]
Zorbax SB-C_{18}[g], 4.6 × 150 (3.5 µm); 30°C	MeOH–0.15% HOAc (25:75 → 90:10)	1.00	UV	Wulfenoside, globularin, isoscrophularioside; *Wulfenia carinthiaca*	[122]

TABLE 28.2 (CONTINUED)
Examples of Analytical HPLC of Iridoids

Column[1]	Mobile Phase[2]	Flow Rate[3]	Detection[4]	Compounds and Plants	Ref.
LiChrospher 60RP select B[c], 4 × 250 (5 µm)	MeCN–1% HCO_2H–TCA (10:100:0.25, v/v/w)	1.2	UV TSP-MS	Loganin, secologanin; *Catharanthus roseus*, *Tabernaemontana divaricata*	[99,100]
Luna C_{18}[f], 4.6 × 150 (5 µm); 30°C	MeOH–H_2O (10:90 → 50:50)	1	UV ESI-MS	Sesamoside, lamiol, shanzhiside methyl ester, lamioside, barlerine, caryoptoside; *Lamium* spp.	[84]
Polaris TM C18-E[h], 4.6 × 250; 26°C	MeCN–H_2O (pH 3.2, formic acid) (13:87 →→ 95:5)	0.8	UV ESI-MS	Verbenalin, hastatoside; *Verbena officinalis*	[123]
Symmetry[b], 3.9 × 250 (5 µm)	MeOH–H_2O (28:72 →→ 100:0)	1	UV ESI-MS	Aucubin 6-*O*-acylrhamnosides; *Jamesbrittenia fodina*	[97]
Zorbax SB-C_{18}[g], 4.6 × 150 (3 µm); 22°C	MeOH–H_2O, both with 0.15% HOAc (25:75 →→ 90:10)	1	UV ESI-MS	Picroside-I, picroside-II, veronicoside, minecoside; *Picrorhiza kurroa*	[109]
LiChrospher 100RP[c], 4 × 250 (5 µm); ambient temp.	MeCN–H_2O (both with 0.1% formic acid) (0:100 →→ 100:0)	—	ESI-MS	Aucubin, catalpol, harpagoside; *Scrophularia nodosa*	[119]
Nova-Pak RP-18[b], 3.9 × 150 (4 µm)	MeCN–aq. 0.05% TFA (5:95 → 65:35)	1	UV TSP-MS ESI-MS	Corniculoside; *Halenia corniculata*	[104]
Nova-Pak RP-18[b], 3.9 × 150 (4 µm)	MeCN–aq. 0.05% TFA (5:95 → 65:35) (D_2O in LC–NMR)	1	UV TSP-MS ^{1}H-NMR	Seemannoside B; *Lisianthius seemannii*	[95]
Luna Aqua[f], 4.6 × 150 (5 µm)	Aq. HCO_2H (0.1%)	0.5	UV ESI-MS ^{1}H-NMR	Monotropein and derivatives; *Vaccinium* spp.	[94]
Nucleosil[e], 8 × 250 (5 µm)	MeCN–D_2O	1	UV ^{1}H-NMR	Aucubin 6-*O*-acylrhamnosides; *Jamesbrittenia fodina*	[97]
Alltima C_{18}[a], 4.6 × 125 (5 µm); 26°C	MeOH–H_2O–HOAc (30:70:0.5)	1	ELSD	Loganin, 7-epiloganin, sweroside, secoxyloganin; *Lonicera* spp.	[91]
Amino phases (NH2)					
Zorbax-NH_2[g], 4.6 × 250 (5 µm); 20°C	MeCN–H_2O (78:22)	0.5	ELSD	Picroside-I, picroside-II; *Picrorhiza* spp.	[75]
Discovery RP[i], 4.6 × 250	MeCN–H_2O (15:85)	1	—	Prismatomerin; *Prismatomeris tetrandra*	[76]

(*Continued*)

TABLE 28.2 (CONTINUED)
Examples of Analytical HPLC of Iridoids

Column[1]	Mobile Phase[2]	Flow Rate[3]	Detection[4]	Compounds and Plants	Ref.
Ion (anion)-exchange columns					
Carbopac PA1[j], 4 × 250	1M NaOH–H_2O	1	EC	Aucubin, catalpol; *Plantago lanceolata*	[77]

[1] Brand, diameter and length (mm), particle diameter, and operating temperature where reported. Column manufacturers: a, Alltech; b, Waters Associates; c, Merck; d, Hewlett Packard; e, Macherey-Nagel; f, Phenomenex; g, Agilent Technologies; h, Varian; i, Supelco; j, Dionex.

[2] Mobile phase components in volume ratio, unless otherwise stated. An arrow (→) denotes a linear gradient; double arrows (→→) denote sectioned (discontinuous, multistep) gradients.

[3] In mL/min.

[4] EC, electrochemical (pulsed amperimetric); ELSD, evaporative light scattering detector; ESI-MS, electrospray ionization–mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; TSP-MS, thermospray mass spectrometry; UV, ultraviolet absorption, including diode array detection.

[5] Not reported.

separations due to a higher solvent flow rate. Schmidt has compared the efficiency of a chromolish RP-18e column (4.6 × 100 mm) to that of the conventional particle-packed Hypersil ODS (4 × 125 mm; 5 μm) in the separation of the iridoids in *Harpagophytum* [78].

28.4.2 Mobile Phases

The choice of the mobile phase and elution program is based on the column chemistry, polarity of the analytes, and complexity of the sample mixture. In reversed-phase chromatography, acetonitrile and methanol are the most commonly used as organic modifiers. Acetonitrile is less viscous and has greater eluting power than methanol. It interferes less in the shorter wavelength region, which makes it suitable for analysis of weakly UV-absorbing compounds. Høgedal and Mølgaard have chosen acetonitrile for monitoring antirrhinoside and its derivatives in *Antirrhinum majus* at UV 205 nm [49]. However, the organic component had to be reduced to 3% to compensate for its eluting power.

Isocratic conditions have often been used in iridoid analyses, and the percentage of organic modifier was determined by the general polarity of the analytes. Methanol and water at a ratio of 3:7 was used for separation of secoiridoid glucosides from *Gentiana lutea* [79,80], while a 7:3 mixture was used for elution of more lipophilic nonglycosidic acyliridoids from *Valeriana officinalis* [81]. Tertial solvent systems consisting of MeCN–MeOH–H_2O and MeCN–THF (tetrahydrofuran)–H_2O were used on C_{18} phases in isocratic mode for separation of harpagosides [82] and the iridoids of the Rubiaceae, gardenoside and geniposide [83]. The distinct advantages of using isocratic elution are the shorter run times and minimal baseline drift. However, the samples should be sufficiently clean and devoid of late-eluting components or coelution of contaminants may occur during the subsequent runs.

Gradient elution is required when the sample mixture is complex, as is often the case with unprocessed crude plant extracts. The initial composition of the solvent and the steepness of the gradient are chosen to accommodate compounds of wide-ranging polarity within a reasonable elution time, typically within a retention factor (*k'*) range of 2–20. The change in the solvent composition can be linear or sectioned (multistage linear gradient). Alipieva et al. have used a multistage gradient of MeOH–H_2O on a Luna C_{18} column (150 × 4.6 mm; 5 μm) to separate nine iridoids of different classes (C_9 and C_{10} iridoids) [84]. Similar approaches were also applied for separation of iridoids

and secoiridoids from *Gentiana triflora* [73], *Scrophularia scorodonia* [85], *Centaurium erythraea* [86], and *Oldenlandia* spp. [87].

Addition of a small amount of acid often improves chromatographic separation and in some cases affects the detection of compounds. Thus, the presence of 0.02% phosphoric acid in an MeOH–H_2O (15:85) mixture was shown to provide a better separation of geniposidic acid and asperulosidic acid, compared to a neutral solvent system [88]. Other reported cases of using small amounts of acid in the mobile phase are listed in Table 28.2. Aberham et al. have explored a number of chromatographic parameters, including the presence of acetic acid, formic acid, or trifluoroacetic acid in the mobile phase, for simultaneous detection (UV 232 nm and electrospray ionization–mass spectrometry [ESI-MS]) of iridoids, secoiridoids, and xanthones in *Gentiana lutea* [50]. The optimal condition was found to be a sectioned gradient program comprising MeCN–*n*-propanol (1:1) and 0.025% trifluoroacetic acid in water.

Ion-pairing agents have been used to induce the retention of highly polar iridoids in reversed-phase HPLC. For example, monotropein, geniposidic acid, and deacetylasperulosidic acid were separated on a μBondapak C_{18} column with a relatively high content of organic modifier in the mobile phase (20% MeOH) and 5 mM tetrabutylammonium phosphate (pH 7.5) as an ion-pairing agent [89]. Tetrabutylammonium chloride was used at 1 mM in an analysis of cornin and dihydrocornin in *Cornus capitata*, with a gradient elution of 10:90–80:20 MeCN–H_2O, on a Tosoh ODS-120A column (4.6 × 250 mm) [90].

28.4.3 Detectors

UV, including photodiode array (PDA) detectors, are routinely used where the target analytes have a suitable chromophore (Table 28.2). Evaporative light scattering detectors (ELSD), suitable for analyses of nonvolatile compounds, have been used in both qualitative and quantitative analyses of iridoids [75,91]. This technique requires all components of the mobile phase to be completely volatile; therefore, phosphoric acid, for example, cannot be used as a buffering agent.

Mass spectrometry (MS) is a detection technique increasingly used in HPLC analyses of iridoids. It provides certain structural information and allows confirmation of the identity of the eluted compounds. Earlier interfaces for direct coupling of HPLC to a mass spectrometer were thermospray (TSP) and continuous flow-fast atom bombardment (CF-FAB) ionizations. Thus, rhodenthoside A was detected with TSP-MS in positive mode and with CF-FAB-MS in negative mode after separation on a Novapak RP-18 column (3.9 × 150 mm, 4 μm) with an MeCN–H_2O gradient [92]. Ammonium acetate (0.5 M) and glycerol were added postcolumn for TSP-MS and CF-FAB-MS detection, respectively, to facilitate the ionization of the compounds.

Electrospray ionization (ESI) mode is suited for analysis of relatively polar compounds and is the most commonly employed technique in recent years. It allows direct introduction of the HPLC effluent into the detector, usually at a flow rate up to 1 mL/min. Both positive and negative modes are used in analysis of iridoids. The positive mode often gives ion peaks corresponding to sodium and potassium adducts ($[M + Na]^+$, $[M + K]^+$) in addition to the protonated molecular ion $[M + H]^+$, which facilitates determination of the molecular weight. Anionic species, such as acids and those with free phenolic hydroxy groups such as caffeic acid derivatives, are more readily detected in the negative mode as the deprotonated molecular ion $[M\text{-}H]^-$. In a chemosystematic survey of *Lamium* spp., a combination of positive and negative ions and cluster ions, for example, $[2M + H]^+$, as well as the retention times of iridoids, were considered together to secure the identification of the analytes [84]. A high-resolution mass spectrometer based on time-of-flight (TOF) was used in combination with an ESI source to determine the elemental composition of compounds isolated from *Veronica* spp. [93].

The most powerful setup for structure determination of unknown compounds in a sample mixture is a coupling of HPLC with a nuclear magnetic resonance (NMR) spectrometer, with or without a UV detector in between. Both LC-MS and LC-NMR techniques were used for preliminary detection

and characterization of iridoids, which were subsequently isolated with preparative HPLC and their structure confirmed by off-line NMR [94]. Rodriguez et al. have used HPLC-NMR to determine the structure of seemannosides in *Lisianthus seemanii* [95]. In these compounds the *p*-coumaroyl group spontaneously isomerizes between *cis*- and *trans*-forms, and these could not be isolated individually. Their structures were determined on-line by stopped-flow LC-^{1}H NMR, using a C_{18} column with MeCN–deuterium oxide as eluting solvent. The natural occurrence of both forms was also confirmed by LC-UV-MS [95]. Another example for LC-NMR application is the analysis of a series of cinnamic acid derivatives of 6-*O*-rhamnosylcatalpol in *Gmelina philippensis*. Helfrich and Rimpler have found that, in solution, the cinnamic acid moieties were migrating between 2-, 3-, and 4-OH positions of the 6-*O*-rhamnose [96]. They were unable to isolate the individual isomers but could reduce the transesterification by immediate processing of fractions by freezing under liquid nitrogen followed by lyophilization. In a separate study, the migration of cinnamoyl groups on the rhamnose could be observed in situ by coupling HPLC and stopped-flow NMR with a 60 μL ^{1}H [^{13}C] flow probe [97].

28.4.4 Applications: Qualitative and Quantitative Analysis

The main applications of the analytical HPLC methods for iridoids are the qualitative and quantitative analysis of plant extracts. Quality control and authentication of herbal medicines is an area where HPLC-based analysis is often adopted. The commercially produced herbal medicines are generally in the form of a powder or extract, and morphological identification of the plant source is not feasible. The presence of marker compounds unique to the species could demonstrate the botanical origin of the formulations and help identify contaminated (adulterated) material. HPLC methods were developed to discriminate the proper from adulterant species of *Gentiana* [73], *Oldenlandia* [87], and *Harpagophytum* [98]. Dagnino et al. proposed an authentication method for *Catharanthus roseus* and *Tabernaemontana divaricata*, analyzing precursors of terpenoid-indole alkaloids with a C_{18} column and a solvent mixture of MeCN–1% aqueous formic acid–trichloroacetic acid (10:100:0.25) [99]. A method employing PDA and TSP-MS detectors (limit of detection 2 nmol/g dry cell) was used to establish the subcellular location of loganin and secologanin (Figure 28.3, 6), their biosynthesis and accumulation in the vacuoles of the cultured cells of *Catharanthus roseus* [100].

HPLC-based analysis has also been used to examine the formation of artifacts during isolation of iridoids, when structures such as methyl acetal and esters were isolated. For example, secoxyloganin dimethylacetal, isolated from *Kissenia capensis*, could not be detected by HPLC-MS when the plant was extracted with acetone [101]. It was assumed to be an artifact derived from morroniside, formed when MeOH was used as the extraction solvent. On the other hand, the natural occurrence of a methyl acetal in *Vitex cymosa* was confirmed after re-extraction of the plant without using MeOH, followed by HPLC analysis [102].

Qualitative HPLC analyses have been used to assist with the structural elucidation of compounds. A library of data on UV and mass spectra, and retention times of well-dispersed peaks in analytical HPLC, is a basic requirement for these strategies. Bergeron et al. subjected an isolated, unknown iridoid glycoside to enzyme hydrolysis, and the degradation products were separated and identified with HPLC as the known trifloroside and deglucosyltrifloroside [103]. Together with MS and NMR data, the unknown iridoid was identified as the higher homologue, 6′′′′-*O*-glucosyltrifloroside. Rodriguez et al. have determined the structure of the secoiridoid corniculoside after detecting 4-*O*-glucosylcaffeoyl and 7-hydroxysweroside moieties following enzyme, acid, or alkaline hydrolyses [104]. The time course of alkaline hydrolysis was determined by periodical sampling of the reaction mixture followed by injection without purification to an HPLC-UV system.

In the past, methods for quantitative analysis of iridoids included gas chromatography (GC) following derivatization to volatile forms [105], and TLC-densitometry [106]. However, modern HPLC techniques superseded these methods because of their higher sensitivity and resolution. Quantitative analysis requires a linear detector response to the analyte concentration; therefore, UV absorption has been the method of choice as the most reliable and convenient. Detection wavelengths

are selected so that the readings are not affected by the baseline drift due to gradient elution, for example, 281 and 328 nm for phenylpropanoid esters [107] or, in the case of weakly UV-absorbing compounds, by using isocratic mode [49,79,80]. ELSD has also been used, especially for poorly UV-absorbing compounds. Although the sensitivity of this detector is lower than that of UV detectors, the response is quasiproportional to the absolute quantity of the analytes regardless of the molecule's spectral properties. The limits of detection and quantitation for two derivatives of catalpol detected by ELSD in *Picrorhiza* spp. were reported as 0.19 and 0.68 μg (for picroside I) and 0.29 and 0.87 μg (for picroside II), and the linearity range was within 30–300 μg/mL [75]. An electrochemical detector was used for quantitative analysis of aucubin and catalpol in leaves of *Plantago lanceolata*, following an ion-exchange mode of separation [77].

28.4.5 Comparison of HPLC with Other Techniques in Iridoid Analysis

The efficiency of HPLC systems has been cross-examined against different chromatographic systems. Bicchi et al. compared C_{18} HPLC with packed-column supercritical fluid chromatography (p-SFC) and found that HPLC offered higher resolution of valepotriates but with a longer run time (40 min) than p-SFC (less than 20 min) [108]. In the analysis of *Picrorhiza kurroa*, the HPLC run time was 40 min, while micellar electrokinetic chromatography (MEKC) took less than 15 min for an equivalent separation [109]. In a comparison of reversed-phase HPLC and normal-phase high-performance thin-layer chromatography (HPTLC) with chromogenic spray and densitometric determination for quantitation of verbenalin and hastatoside, the HPLC method gave better baseline separation and higher resolution [110]. However, in a quantitative analysis of highly polar compounds that are poorly retained on a reversed phase, such as aucubin and catalpol, HPTLC on normal phase was found to give better results with smaller standard deviations [111].

28.5 ADVANCED AND SPECIALIZED HPLC SETUPS

In recent years there has been a remarkable development in the physical format of the columns and stationary phases, as well as highly specialized organization of HPLC components. Some special HPLC setups used for iridoid isolation and analysis are discussed in the following.

Capillary HPLC methods use particle-based stationary phases packed into capillary tubing with an inner diameter under 1 mm. The iridoids and secoiridoids of *Lonicera* spp. were separated on a Zorbax SB-C_{18} column (0.3 mm ID × 150 mm; 5 μm), with MeOH–MeCN–aqueous acetic acid at a flow rate of 5 μL/min and detected by ESI-MS in negative mode [112]. The very low flow rate eliminated the need for a splitter, a source of uncertainty in a quantitative analysis, and gave a greater sensitivity due to higher ionization efficiency in ESI. The same research group later introduced a UV detector (250 and 350 nm) for simultaneous analysis of flavonoids and saponins in addition to iridoids [113]. In a chemotaxonomic survey of valepotriates in the Valerianaceae and allied families, capillary HPLC was coupled to a FAB-mass spectrometer [26]. In order to obtain a flow rate of 4 μL/min the eluent from pump (250 μL/min) was split to the capillary column (LC Packings Fusica-C, C18, 0.32 × 300 mm; 5 μm) and a narrow bore column (Nucleosil C18, 2.1 × 120 mm; 3 μm), the latter providing the system with a backpressure, ensuring the solvent flow to the capillary column. Samples were introduced through a loop with a capacity of 500 nL. Ionization of the compounds was assisted by the addition of 1% glycerol to the eluent (MeOH–1% aqueous acetic acid, 7:3).

Ultrahigh-pressure liquid chromatography (UHPLC) uses columns packed with sub-2-μm particles. The theoretical plate numbers are increased, and shorter columns (50–100 mm) can achieve comparable separation to conventional-sized columns. Mass spectrometers are routinely used as the detector, often coupled with a UV detector. Twelve iridoids were detected in *Hedyotis diffusa* using UHPLC with UV and tandem mass spectrometric (MS/MS) detectors [114]. Six compounds were determined by comparison with standard samples, and the structures of the remaining six were tentatively elucidated by analysis of high-resolution MS/MS fragmentation patterns.

Two-dimensional HPLC enables isolation of plant secondary metabolites of similar structures and polarity, using two columns and separation conditions independent to each other. Thus, shanzhiside, gardenoside, and methyl esters of deacetylasperulosidic acid and scandoside were purified after injection of a crude 50% ethanol extract of *Gardenia jasminoides* into an HPLC system comprising two YMC C_{18}-phase columns of different sizes [115]. These compounds were insufficiently separated on the first column (10 mm ID × 100 mm), but the effluent containing the four iridoids (elution time 1.5–6.5 min) was introduced directly to and successfully separated on the second column (10 mm ID × 250 mm). Both dimensions were operated isocratically in the same solvent system, MeCN–H_2O–acetic acid (7:93:0.1).

Multichannel parallel HPLC uses corresponding numbers of identical pumps, columns, and detectors controlled by a single computer system; for example, an eight-channel parallel analytical and a four-channel preparative HPLC system have been constructed [116]. Six known compounds were isolated from a prefractionated extract of *Penstemon centranthifolius*, and the structures were subsequently determined with capillary NMR equipped with a 5 μL flow cell [116].

Recycle preparative HPLC was used by Takasaki et al., in a rare example, for isolation of iridoids from *Ajuga decumbens* [117]. A JAIGEL 310 system equipped with a 20 mm ID × 500 mm column and MeOH as the eluent was used for the final purification of reptoside, harpagide, and its 8-*O*-acetyl derivative. The details of the HPLC column chemistry and operating conditions, however, were not reported in the literature.

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Secondary Metabolites — Amino Acid Derivatives

29 HPLC of Indole Alkaloids

Anna Petruczynik

CONTENTS

29.1 DEFINITION, CHEMISTRY, AND CLASSIFICATION OF INDOLE ALKALOIDS

Indole alkaloids constitute an important class of natural products that includes a large number of pharmacologically important substances such as antitumor alkaloids (vinblastine, vincristine, reserpine, and ajmaline), blood pressure lowering substances (reserpine), an alkaloid (ajmaline) used to treat cardiac arrythmias, and the hallucinogen lysergic acid and its derivatives. Some of the indole alkaloids are strongly toxic (strychnine) or psychoactive substances (psylocibine, bufotenin). These compounds belong to one of the largest groups of secondary metabolites, comprising different structures often with remarkable complexity. Indole is an aromatic heterocyclic organic compound (Figure 29.1). It has a bicyclic structure, consisting of a six-membered benzene ring fused to a five-membered nitrogen-containing pyrrole ring. Indole alkaloids are biogenetically derived from tryptophan. These alkaloids contain two nitrogen atoms, one of which is contained within the five-membered part of the indole nucleus. Alkaloids such as quinine and camptothecin and its derivatives, with a five-membered heterocylic ring that is expanded by the inclusion of an extra carbon, are classified as quinolines rather than indoles, although their biogenic origins are the same. The diversity of alkaloid structures forced scientists to concentrate during recent years on the elucidation

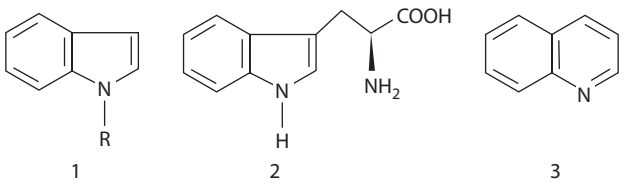


FIGURE 29.1 Structure of (1) indole, (2) tryptophan, and (3) quinoline.

of biosynthetic pathways at the enzymatic level. Thus, during the last few years, a highly successful study of the molecular genetics of alkaloid formation has been undertaken, including the first examples of heterologous expression of appropriate enzymes catalyzing alkaloid metabolism.

Usually all the alkaloids occur in multicomponent mixtures, and separation of alkaloids from other groups of natural products is the first requirement for detailed qualitative, quantitative, and structural analysis of single alkaloids. One of the most effective methods for the analysis of alkaloids is high performance liquid chromatography (HPLC) because of its high resolution power and automatization. Table 29.1 presents the structures of the indole alkaloids.

29.2 OCCURRENCE AND MEDICAL SIGNIFICANCE OF INDOLE ALKALOIDS

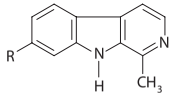
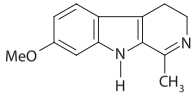
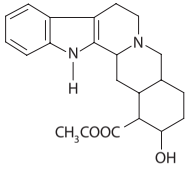
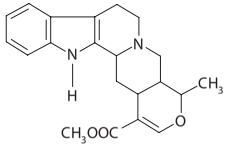
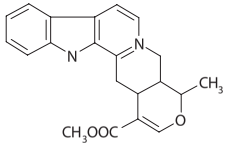
Indole alkaloids belong to one of the largest groups of secondary metabolites and comprise various structures. Many of them exhibit important biological and pharmacological activities and have been therapeutically applied for several decades in the treatment of various diseases [1]. The biological activity of some plants (*Rauvolfia serpentina*) and fungi (*Claviceps purpurea*) has been known for a long time. Indole alkaloids such as ajmaline, reserpine, yochimbine, and others, which are contained in *Rauvolfia serpentina* family, are biologically active substances, valuable drugs and semiproducts of drugs [2,3]. *Rauvolfia serpentina* is a shrub common to India that has been used as a panacea in the Ayurvedic system of medicine, with uses described for the treatment of snakebite and madness [4]. Reserpine, the major component of this species and the most widely used alkaloid from it, was the first tranquilizing drug and was also used as an antihypertensive agent. Due to side effects (neurotoxicity, cytotoxicity, and depression), however, it is no longer the drug of choice. Many indole and dihydroindole alkaloids from this plant have served as leads in the development of novel drugs for the treatment of cardiovascular diseases. Ajmaline is used to treat cardiac arrhythmia.

The pentacyclic oxindole alkaloids contained in *Uncaria tomentosa* are used as biochemical markers for cat's claw and are responsible, in part, for its immunomodulatory, cytotoxic, anti-AIDS, and anti-amyloidosis activities [5,6]. For example, they inhibited the growth of HL60 and U-937 leukemic cells [7]. Selectivity between leukemic and normal cells was observed [8]. Recently, medical preparations from *Uncaria tomentosa* have become very popular in Europe and America, particularly as anti-cancer remedies. The indole alkaloids from *Himatanthus sucuuba* have anti-inflammatory and analgesic activities [9].

Catharanthus roseus is one of most extensively investigated medicinal plants. It contains a very large number of alkaloids, about 100 of which have been isolated so far [10]. *Catharanthus roseus* produces such alkaloids as vinblastine and vincristine, which are widely used to combat a number of different cancers [10–12]. They exert their anti-cancer effects by inhibiting mitosis through binding to tubulin, thus preventing the cell from making the spindles it needs to be able to move its chromosomes around as it divides [4]. Ajmalicine and serpentine are other medicinally valuable alkaloids from *C. roseus*; they are used as antihypertensive agents [13]. Catharanthine, tabersonine, horhammericine, lochnericine, and other indole alkaloids have also been found in *C. roseus* plant extract [10,14].

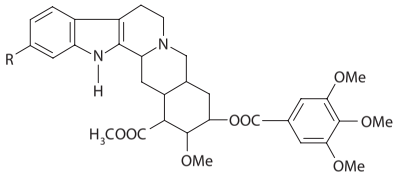
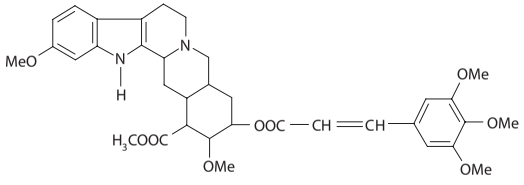
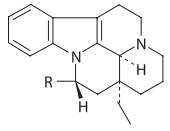
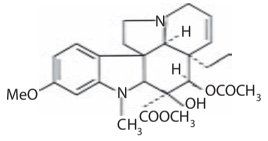
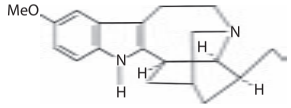
Alstilobianines contained in *Alstonia angustiloba* showed a slow relaxation effect [15]. Vincamine is an alkaloid derived from *Vinca minor* plant, which has been used for the prevention

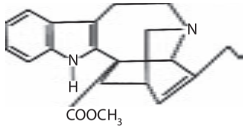
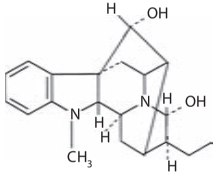
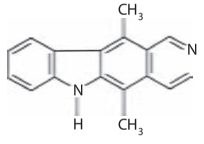
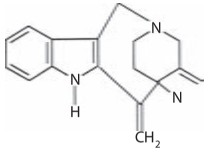
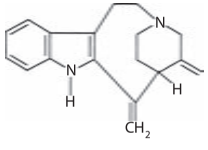
TABLE 29.1
Structures of Indole Alkaloids

Type	Structure	Substituents	Alkaloid	Plant Raw Materials	Activity
β-Carbolines		R = H	Harmane	*Passiflora* sp.	Antileishmanial, neuronal activity
		R = OCH_3	Harmine	*Passiflora incarnata*, *Peganum harmala*	Antitumor, neurotoxic, antiplasmodial
			Harmaline	*Peganum harmala*, *Banisteriopsis caapi*	Central nervous system stimulant, specific neuronal activity
Yohimbine type			Yohimbine	*Pausynistalia yohimbe*, *Corynanthe yohimbe*, *Pausinystalia yohimb*, *Rauwolfia* sp., *Corynanthe* sp.	Peripheral action spasmolytic
			Ajmalicine	*Catharanthus roseus*, *Vinca rosea*, *Rauwolfia serpentina*	Peripheral action spasmolytic, hypotensive, selective tranquilizing properties
			Serpentine	*Rauvolfia sp.*, *Vinca sp.*	—

(*Continued*)

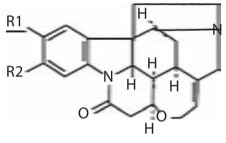
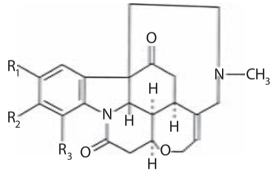
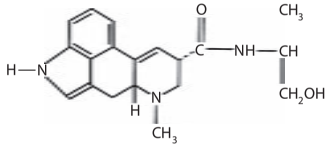
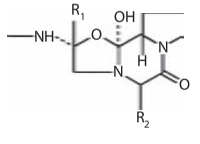
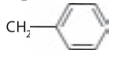
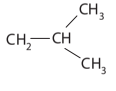
TABLE 29.1 (CONTINUED)
Structures of Indole Alkaloids

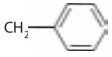
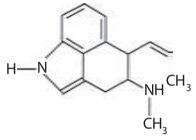
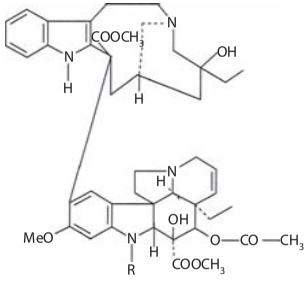
Type	Structure	Substituents	Alkaloid	Plant Raw Materials	Activity
Reserpine type		$R = OCH_3$	Reserpine	*Rauwolfia serpentina*	Spasmolytic, sedative, hypotensive, psychotropic activity, modulates catecholaminergic neuronal function
		$R = H$	Deserpidine	*Rauwolfia serpentina*	Active in mental depression
			Rescinamine	*Rauwolfia serpentina*	
Eburnamine type		$R = OH$	Eburnamine	*Kopsia* sp., *Pleiocarpa mutica*	
		$R = CH_3COO$	Vincamine	*Vinca minor*	Hypotensive, used in cases of central sclerosis and cerebral vascular accidents
Aspidospermine type			Vindoline	*Aspidosperma*, *Vinca*, *Catharanthus*, *Vallesia*	Precursor of dimeric anticancer drugs
Ibogaine type			Ibogaine	*Tabernanthe iboga*	Psychotropic, hallucinogenic

	Catharanthine	*Catharanthus roseus*	—
	Ajmaline	*Rauwolfia serpentina*	Antiarrhythmic
	Elipticine	*Ochrosia eliptica*	Cytostatic
	Picraline	*Picralima nitida*	Analgesic
	Pericine	*Picralima nitida*	Analgesic

(*Continued*)

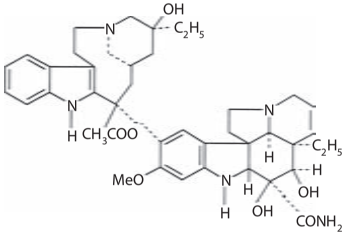
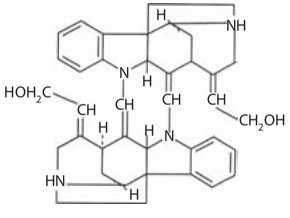
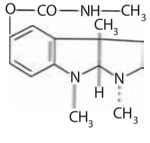
TABLE 29.1 (CONTINUED)
Structures of Indole Alkaloids

Type	Structure	Substituents	Alkaloid	Plant Raw Materials	Activity
Strychnine type		$R_1 = R_2 = H$	Strychnine	*Strychnos nux-vomica*	Analeptic, tonic, strongly toxic
		$R_1 = R_2 = OCH_3$	Brucine	*Strychnos nux-vomica*	Analgesic and anti-inflammatory, cytotoxicity
		$R_1 = H$, $R_2 = OCH_3$	α-Colubrine	*Strychnos nux-vomica*	Neurotoxic activity
		$R_1 = OCH_3$, $R_2 = H$	β-Colubrine	*Strychnosnux-vomica*	Neurotoxic activity
		$R_1 = R_2 = OCH_3$ $R_3 = H$	Novacine	*Strychnos nux-vomica*	—
		$R_1 = R_2 = H$, $R_3 = OH$	Vomicine	*Strychnos nux-vomica*	Convulsant
Secale cornutum alkaloids			Ergometrine	*Claviceps purpurea*	Stimulate uterine contractions, uterotonicum, vasospastic
		$R_1 = CH_3$ $R_2 =$ 	Ergotamine	*Claviceps purpurea*	Spasmolitic, uterotonicum
		$R_1 = CH_3$ $R_2 =$ 	Ergosine	*Claviceps purpurea*	Uterotonicum

		R_1 = 	Ergocristine	*Claviceps purpurea*	Lactation inhibition
		R_2 =  R_1 = R_2 = 	Ergocriptine	*Claviceps purpurea*	Lactation inhibition
		R_1 =  R_2 = 	Ergocornine	*Claviceps purpurea*	Lactation inhibition
Clavine type			Chanoclavine	*Claviceps purpurea*	Psychoactive
Dimeric indole alkaloids		$R = CH_3$	Vinblastine	*Catharanthus roseus*	Cytostatic
		R = CHO	Vincristine	*Catharanthus roseus*	Cytostatic

(*Continued*)

TABLE 29.1 (CONTINUED)
Structures of Indole Alkaloids

Type	Structure	Substituents	Alkaloid	Plant Raw Materials	Activity
			Vindesine	*Catharanthus roseus*	Anticancer
			Toxyferine C	*Strychnos toxifera*	Neuromuscular-blocking activity
Other indole alkaloids			Physostigmine	*Physostigma venenosum*	Inhibitor of acetylcholinesterase

and treatment cerebrovascular insufficiencies and disorders [16]. Vinacamine is claimed to have a selective vasoregulatory action on cerebral circulation, ad adapting cerebral blood flow to metabolic needs [17]. Diprenylated indole alkaloids from *Glycosmis montana* have potential anti-HIV activity [18]. A number of studies have reported different pharmacological effects of *Psychotria* alkaloids, such as inhibition of the aggregation of human platelets, cytotoxicity, and analgestic activity [19–21]. *Camptotheca acuminata* is a tree indigenous to southern China that produces antitumor alkaloids, most notably camptothecin [22]. Camptothecin's anti-tumor activity is due to its inhibition of DNA topoisomerase I [23]. *Nauclea pobeguinii* extract containing inter alia strictosamide is an active antiplasmodial remedy [24].

Physostigma venenosum is one of the numerous woody climbers that inhabit the tropical forests of Africa. Physostigmine, calabarine, eseridine, eseramine, and phytosterin are some of the components of *Physostigma venenosum*. Physostigmine is a powerful sialogogue and stimulates almost all involuntary muscles in the body. It is a powerful and reversible acetocholine esterase inhibitor, which effectively increases the concentration of acetylocholine at the sites of cholinergic transmission. Physostigmine has been used mainly for the treatment of glaucoma and myasthenia gravis, as it is capable of constriction the pupil and hence facilitates the outflow of the aqueous humor [25]. There is interest in this compound in the treatment of Alzheimer's disease, in which a low concentration of acetylcholine in the brain is observed [4]. Rivastigmine, a semisynthetic derivative of physostigmine, is a reversible, noncompetitive inhibitor of acetylcholinesterase.

Monoterpenoid indole alkaloids from an *Aspidosperma ramiflorum* plant extract showed significant activity against *Leishmania amazonensis* [26]. The effect of an indole alkaloid, mitragynine, isolated from the Thai medicinal herb *Mitragyna speciosa* on neurogenic contraction of smooth muscle was studied in guinea-pig vas deferens. Mitragynine inhibited the contraction of the vas deferens produced by electrical transmular stimulation. At the same time, mitragynine failed to affect the responses to norepinephrine and ATP. Mitragynine did not reduce KCl-induced contraction in the presence of tetrodotoxin, prazosin, and α, β-methylene ATP. Mitragynine inhibited nicotine- or tyramine-induced contraction. The leaf of *Mitragyna speciosa* has been used in Thailand for its opiumlike effect and its coca-like stimulant effect to combat fatigue and enhance tolerance for hard work under the hot sun [27].

Many plant species containing indole alkaloids were widely used in tradicional medical systems. *Centaurea montana* has long been used in tradicional medicine to cure various ailments, for example, diabetes, rheumatism, malaria, and hypertension. Dimeric indole alkaloids isolated from the plant material demonstrate cytotoxic activity [28]. Extracts from *Centaurea schischkinii* were introduced to Turkey for the treatment of malaria, diabetes, hypertension, rheumatism, and diarrhea. From *Centaurea schischkinii* extract the indole alkaloid scischkiniin with cytotoxic activity was isolated [29]. Medical preparations from *Uncaria tomentosa* have became very popular anticancer remedy [30].

Many psychoactive compounds such as harmine and harmaline alkaloids are structurally related to tryptamine. *Pegnanum harmala*, an herb containing these indole alkaloids (harmol, harmalol, harmine, and harmaline), is native to dry areas from the Mediterranean east to northern India. Extracts from the plant are used in folk medicine as an antihemarrhoidal, helmicide, and central nervous system-stimulating agent [31]. Harmine and related alkaloids are serotonin antagonists, hallucinogens, CNS stimulants, and short-term monoamine oxidase (MAO) inhibitors. Small doses (25 mg) act as a mild and therapeutic cerebral stimulant, sometimes producing a drowsy or dreamy state for 1–2 h. Larger doses up to 750 mg may have hallucinogenic effects, the intensity of which varies widely with the individual. Harmaline is a strong MAO inhibitor and is sometimes used in Parkinson's disease. Harman possesses diverse biological properties (hallucinogenic, tremorogenic, anxiolytic, hypotensive, or cardiovascular actions) due to its capability to bind to benzodiazepine or imidazoline receptors [32]. Ibogaine, from *Tabernanthe iboga*, is a hallucinogen and anticonvulsant and has recently been studied as a treatment for heroin addiction [4]. Its mechanism of action is complex, affecting many different neurotransmitter systems simultaneously. *N*,*N*-Dimethyltryptamine

and 5-methoxydimethyltryptamine are hallucinogenic indole alkaloids that occur naturally in a variety of plants and preparations used by South American cultures to perform shamanistic divination rituals [33].

Poisoning through fungal contamination of rye grain, in particular by *Claviceps purpurea*, has been described since the Middle Ages. This fungus produces structures known as ergot on rye plants; these structures are rich in indole alkaloids. These poisons can cause massive constriction of blood vessels, leading to "blackened" limbs and gangrene [4]. The ergot alkaloids have a high biological activity and a broad spectrum of pharmacological effects, hence they are of considerable importance to medicine. They have adrenoblocking, antiserotonin, and dopaminomimetic properties. Small amide derivatives of lysergic acid are potent and relatively selective antagonists of 5-HT, while the amino acid alkaloids are usually less selective and show similar affinities as blocking agents at α-adrenergic and tryptaminergic receptors. Dihydrogenated derivatives usually have fewer and less intense agonist actions than do parent alkaloids. Although all natural ergot alkaloids have qualitatively the same effect on the uterus, ergonovine is the most active and is less toxic than ergotamine. For these reasons ergonovine and its semisynthetic derivative methylergonovine have replaced other ergot preparations as uterine-stimulating agents in obstetrics. The natural amino acid alkaloids, particularly ergotamine, constrict both arteries and veins. Ergot alkaloids have a therapeutic effect on some forms of migraine, postpartum hemorrhages, and mastopathy and a sedative effect on the central nervous system [34]. Recent studies involving ergot derivatives in the treatment of Parkinson's disease have investigated their effects on dopamine receptors [35]. The similarity of the ergoline ring structure to the endogenous monoamines explains the action of these compounds on dopaminergic, serotonergic and adrenergic receptors. Ergot alkaloids can cause hallucinations, and the hallucinogenic drug of abuse LSD (lysergic acid) is structurally related to these compounds. Ergotamine can be used to treat severe migraine that cannot be controlled with other drugs. However, production of ergot alkaloids has been reported in widely separated taxa, such as the fungal genera *Aspergillus* and *Penicillium*, as well as in the plant family Convolvulaceae [36]. The ergot alkloids ergovaline, peramine, and lolines are produced by endophyte fungal *Lolium perenne* and *Neotyphodium lolii.* Ergovaline is also produced by the endophythic fungus *Neotyphodium coenophialum* [37]. These alkaloids cause animal toxicoses such as ryegrass staggers [38]. *Centaurea cyanus* flawers containing the indole alkaloids moschamine and centycyamine are also toxic [39]. Ergot alkaloids produced by *Neothyphodium* spp. cause neurotoxic effects on grazing or garnivorous vertebrates, peramine is an insect feeding deterrent, and lolines are insecticidal [40].

The following are some few effective treatments for improving memory, especially in dementia. Psychollatine is a monoterpene indole alkaloid produced and accumulated by *Psychotria umbellata* leaves in relatively high amounts. The alkaloid has been shown to display opioid-like analgesic, anxiolytic, anti-depressive, and anti-psychotic activities in rodents. Psychollatine and the crude foliar extract of *Psychotria umbellata* showed a protective effect against oxidative stress in yeast, acting as both antioxidant and antimutagenic agents [41]. Chimonamidine and chimonathidine—indole alkaloids isolated from an extract of *Chimonanthus praecox* seeds—interact with opioid receptors [42,43].

The neurotransmitters serotonin and the related compounds *N*,*N*-dimethyltryptamine and bufotenine, found in various hallucinogenic snuffs, are including in this group as active constituents of the hallucinogenic fungi of the genus *Psilocybe*. Psilocybin and psilocin are naturally occurring indoles found in several mushroom species at concentrations of up to 0.5% and 2%, respectively [44]. Psilocybin is rapidly dephosphorylated to psilocin in vivo, with the latter compound being structurally related to the neurotransmitter serotonin, which gives rise to its comparable human metabolism. This molecular similarity endows psilocin with high affinity for serotonin receptors, which blocks the release of the neurotransmitter, thus giving rise to hallucinogenic effects.

Strychnos nux-vomica seeds have been used to activate the ionic channels, alleviating pain, reducing swelling, and moving the blood in Oriental medicine [45]. This plant has also been known

to increase spinal reflexes and stimulate respiratory and sensory centers of the cerebral cortex. The main constituents of *Strychnos nux-vomica* seeds were known to be alkaloids, such as strychnine, brucine, and vomicine. Strychnine strongly excites the central nervous system. Preparations of nux-vomica were used as a stimulant tonic until the middle of the twentieth century. Larger doses of strychnine are known to be a deadly poison that leads to violent muscular convulsions. However, small doses of this compound can give subjective feelings of stimulation [45]. They are only of historical interest in pharmacy and are now used as research tools [4].

A number of β-carboline alkaloids of the harmane group are naturally present in plants and may occur in many plant-derived foodstuffs and beverages. Cigarette smoking has been identified as a major source of these alkaloids, but diet may contribute to exposure [46,47]. These compounds have an increasing toxicological significance because of their intense biological activity and adverse effects on human health, being related to Parkinson's disease, alcoholism, and cancer [48]. β-Carboline alkaloids of the harmane group were isolated from *Peganum harmala*, *Arariba rubra*, *Grewia bicolour*, *Passiflora incarnata*, *Tribulus terrestris*, *Festuina arundinacea*, *Lolium perenne*, and *Chrysophyllum lacourtianum* [48]. Ayahuasca is a psychotropic plant tea that has traditionally played a central role in the magico-religious practices and folk medicine of indigenous peoples native to the Amazon River basin. It contains β-carboline alkaloids such as harmine, harmaline, harmol, and harmalol with MAO-inhibiting properties, which could explain the oral psychoactivity of the tea [49].

The production of secondary metabolites by plant cells and tissues has become an active field of study because of their potential as valuable pharmaceutical compounds. In vitro cultures of plant cells or tissues look promising for the large-scale production of secondary metabolites such as indole alkaloids. These cultures are not exposed to disease and pests and seem not to be subject to seasonal and somatic variations [14]. The antitumor *Catharanthus roseus* alkaloids are produced in trace amounts (0.0003% of dry weight). The high prices of these anticancer products have led to a widespread research interest in the development of alternative sources for these alkaloids [10]. *Catharanthus roseus* cell cultures can be used for the production of therapeutically important alkaloids, such as catharanthine, ajmalicine, and serpentine [50–54]. *Catharanthus roseus* cell cultures have been studied extensively, but the clinically important anticancer medicine vinblastine still cannot be produced by this technique [55]. The low yield of these substances in the cell cultures is one of the major limitations, and many methods have been tried to improve indole alkaloid production [50].

It is widely believed that the synthesis of secondary metabolites such as indole alkaloids in plants is part of the plants' defense responses to pathogenic attack. An extract of pathogenic microorganisms has, therefore, been used as one of the most effective strategies for improving the production of useful secondary metabolites in plant cell cultures [50,55]. Since selection and maintenance of high-alkaloid-yielding cell lines are laborious and also risk genetic variance during the maintenance of the cell line, the use of pathogenic or nonpathogenic fungal preparations or chemicals, termed elicitors, becomes one of the most important strategies to improve indole alkaloid production in cell cultures [50,52,56–59]. For example, a cell suspension culture was treated with 70% ethanol to increase alkaloid production (Figure 29.2) [1]. Camptothecin and its related compounds are obtained by extraction from plants such as *Ophiorrhiza pumila*, *Camptotheca acuminata*, *Ervatamia heyneana*, and *Nothapodytes foetida*, but transformed plant cell cultures may be an alternative source for it [22,60]. The potential of strategies for increasing secondary metabolite accumulation from plant cell cultures may still be partially limited by unfavorable conditions for biosynthesis (that is, feedback inhibition, unfavorable equilibrium state) or product metabolism [61].

Hairy root cultures represent an alternative to cell suspension cultures for production of secondary metabolites [62]. The advantages of hairy roots in comparison to cell culture systems include their apparent genotypic and biochemical stability, morphological differentiation, and growth in hormone-free media. The hairy roots culture system was applied to production of indole alkaloids

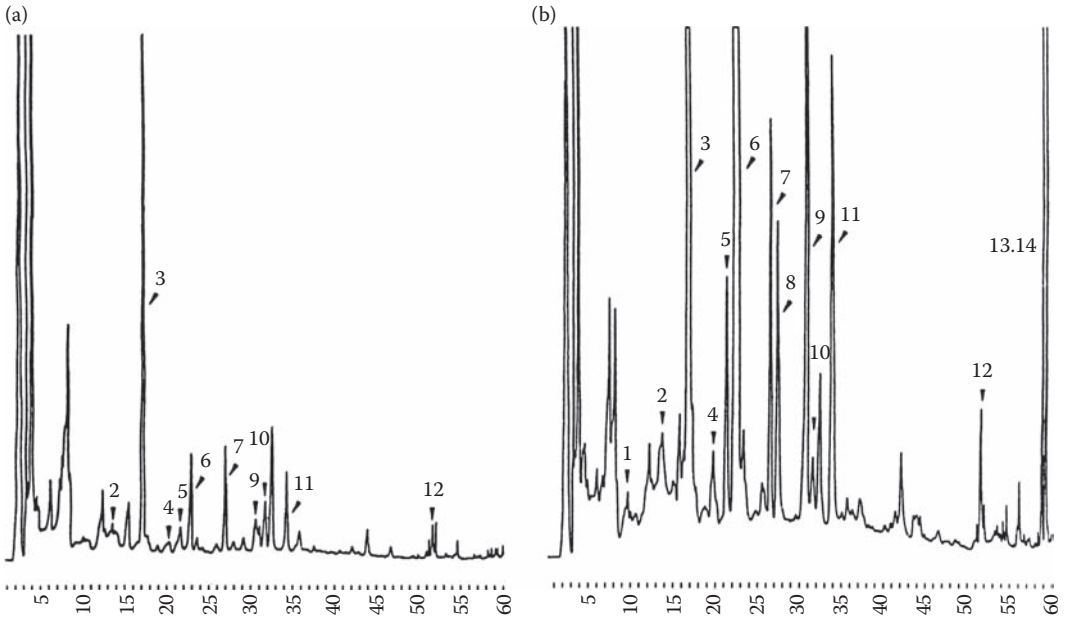


FIGURE 29.2 HPLC separation of the extracts of *Rauvolfia serpentina* X *Ravolfiastricta* hybrid cell cultures (line R X R17M): (a) control and (b) methy jasmonate (MJ) treated cultures on the fifth day after treatment. The major alkaloids were identified: (1) sarpagine; (2) perakine, norajmalie; (3) raucaffricine; (4) ajmaline; (5) vomilenine; (6) strictosidine; (7) an unidentified alkaloid with MW 410; (8) 17-*O*-acetyl-noramalne; (9) strictosidine lactam; (10) ajmalicine; (11) 17-*O*-acetylajmaline; (12) reserpine; and (13, 14) isomers of vallesiachotamine. Chromatographic analysis was performed on a Nucleosil 100-5 C18 column. Gradient elution: 15–20% acetonitrile in a mixture containing 39 mM NaH_2PO_4 and 2.5 mM hexanesulfonic acid within 5 min; 20–40% acetonitrile in a mixture containing 39 mM NaH_2PO_4 and 2.5 mM hexanesulfonic acid within 40 min; and 40–80% acetonitrile mixture containing 39 mM NaH_2PO_4 and 2.5 mM hexanesulfonic acid within 15 min. (From Stöckigt, J., Sheludko, Y., Unger, M., Gerasimenko, I., Warzecha, H., and Stöckigt, D., *J. Chromatogr. A*, 967, 85–113, 2002. With permission.)

from *Catharanthus roseus* [63,64]. A hairy root culture of *Ophiorrhiza pumila* has been established by infection with *Agrobacterium rhizogenes* [65].

The alkaloid content in different species, varieties, and forms may contribute to their better distinction and to better understanding of the taxonomy of the whole genus. Figure 29.3 present differences in chromatograms obtained for *Uncaria tomentosa* and *Uncaria guianensis* plant extracts [66].

29.3 SAMPLE PREPARATION FOR HPLC ANALYSIS

Various methods are used for extraction of indole alkaloids from plant material. Before the extraction procedure the plant tissue should usually be reduced into small particles in a grinder or homogenizer. Generally, in liquid–solid systems acidic extractants have been used. The following mixtures of solvents have been used: methanol (MeOH) with 5% or 2% hydrogen chloride (HCl) [18,67], MeOH with 1% acetic acid (CH_3COOH) [68], H_2O with 0.1% trifluoroacetic acid (TFA) [10], *n*-hexane with 1% HCl [69], MeOH–H_2O with 1% HCl [70], 2-propanol–H_2O with 1% lactic acid [71], MeCN–H_2O with 1% CH_3COOH [72], water with 10% acetic acid [37], and water with 2% sulfuric acid [30].

Indole alkaloids from *Rauvolfia serpentina* were extracted with a nonaqueous mixture of *n*-pentane, dichloromethane (CH_2Cl_2), and MeOH. After evaporation of the solvents, the methanolic

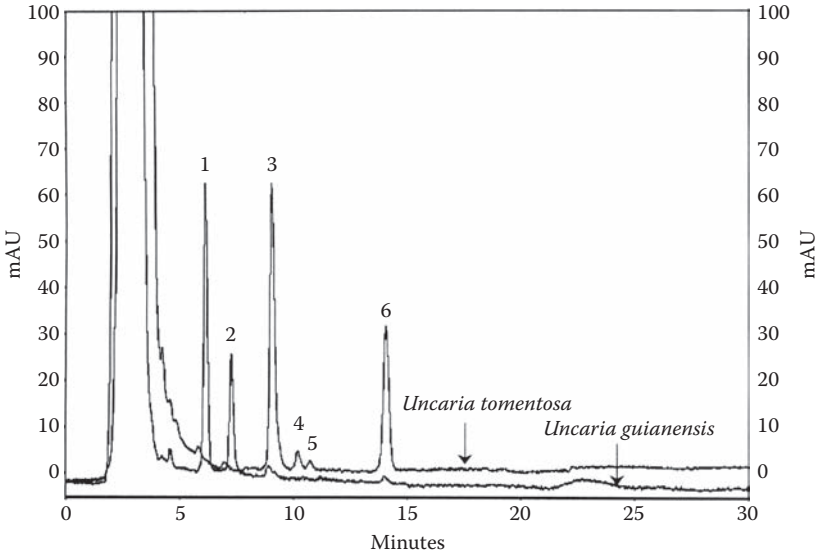


FIGURE 29.3 HPLC chromatogram of freeze-dried cat's claw: *Uncaria tomentosa* and *Uncaria guianensis.* Identified compounds: (1) Speciophyline; (2) mitrphyline; (3) uncarine F; (4) pteropodine, (5) isomitraphylline, and (6) uncarine E. Chromatographic analysis was performed on a C18 column with a mobile phase containing 45% acetonitrile and phosphate buffer at pH 7.0. (From Sandoval, M., Okuhama, N.N., Zhang, X.-J., Condezo, L. A., Lao, J., Angeles, F. M., Musah, R. A., Bobrowskai, P., and Miller, M. J. S., *Phytomedicine*, 9, 325–337, 2002. With permission.)

residue was redissolved in water, and the pH adjusted to 7 using sodium hydroxide (NaOH). This solution was extracted with dichloromethane to remove residual amounts of alkaloids with weak and medium basicity. To extract the highly basic alkaloids, the pH of the aqueous solution was adjusted to 10.5 and further extracted with dichloromethane [73].

The extraction of indole alkaloids was also performed without addition of acids, for example, extraction with pure MeOH [11,14,22,36,40,45,60,61,74–77], ethanol (EtOH) [26,75,78–82], dichloromethane [22], acetone [22], or MeOH–H_2O [12]. Sometimes extraction was performed using an extractant at a basic pH, such as MeOH–H_2O or ethanol with the addition of ammonia [46,83].

For isolation of ajmalicine from *Nauclea pobeguinii*, plant material was exhaustively macerated and percolated with 80% ethanol. After drying, the crude extract was dissolved in water with 2% HCl. After washing with chloroform, the water layer was brought to a pH of 9.0 with 10% ammonia and extracted again with chloroform [24]. The macrolactam-type indole alkaloids from *Ipomoea obscura* were extracted with MeOH. The solvent was evaporated under reduced pressure at 40°C, the residue redissolved in 2% aqueous tartaric acid, and extracted with ethyl acetate. After evaporation of the organic solvent, the residue was dissolved in MeOH [76]. The leaves of *Alstonia angustiloba* were extracted with MeOH, and the extract was partitioned between ethyl acetate and 3% aqueous tartaric acid. Water-soluble materials, which were adjusted to pH 9 with saturated sodium carbonate (Na_2CO_3), were extracted with chloroform [15]. The indole alkaloids from *Psychotria leiocarpa* were extracted with ethanol. The residue was suspended in water and washed with gasoline [84].

Another procedure for extraction of terpenoid indole alkaloids from *Catharanthus pusillus* has also been applied [85]. The plant material was mixed with absolute EtOH, homogenized, and centrifuged, and the ethanolic extract was transferred into a round-bottom flask. The pellet was reextracted in a similar fashion, and the combined ethanolic phases concentrated to dryness under reduced pressure. For the extraction of vindoline, the plant material was mixed with buffer (composed of glycine,

sodium chloride [NaCl], and NaOH) at pH 10 and dichloromethane. The mixture was homogenized and centrifuged. The organic liquid phase was taken and transferred to a round-bottom flask. The slurry was extracted again by addition of dichloromethane [85].

For isolation of camptothecin, the plant material was extracted with ethanol, and this extract was treated with chloroform and aqueous ethanol to give two phases, the lipophilic chloroform phase being highly active. Extensive partitioning of the extract was tried in separating funnels using the quaternary system chloroform–carbon tetrachloride–methanol–water. Corynanthe-type alkaloids were isolated from *Mitragyna speciosa*. Plant material was moistened with 10% aqueous ammonia and allowed to stand overnight. It was then macerated with ethyl acetate and filtered. The extract was roughly separated by silica gel flash column chromatography. The silica column was eluted with *n*-hexane–ethyl acetate to ethyl acetate–MeOH gradient to give the preliminary fractions [86].

Progressive extraction in a Soxhlet apparatus with *n*-hexane, dichloromethane, and methanol [48] or progressive extraction successively with methanol and 2% HCl was also used [2,31]. The alkaloids from *Camptotheca acuminata* were extracted successivelywith methanol, dichloromethane, and acetone [22]. Alkaloids were extracted from *Centaurea montana*, *Centaurea schischkinii*, and *Centaurea cyanus* with *n*-hexane, dichloromethane, and methanol [28,29,48], from *Glycosmis montana* with chloroform, ethyl acetate, and *n*-butanol [18], and from *Centaurea moschata* with *n*-hexane, chloroform, and methanol [87]. β-Carboline alkaloids from *Cortinarius infractus* were extracted in sequence with methanol, ethanol, and water [75]. For isolation of ergot alkaloids from *Claviceps purpurea*, sclerotia were homogenized with methanol. The homogenate was centrifuged, the procedure was repeated, and the pooled extract was evaporated to dryness.

Extraction of indole alkaloids from *Rauvolfia serpentina* tissue was performed on an SCX cation exchanger [88]. Tissues were extracted with methanol. After filtering the extract was evaporated to dryness. The extract was then dissolved in methanol, transferred into an Eppendorf tube, and acidified with 0.1 M HCl. The acidified solution was extracted with the SCX cation exchanger. After centrifugation the alkaloid fraction was eluted with a mixture of methanol and ammonia. Extraction has often been performed in an ultrasonic bath [14,40,46,60].

Catharanthus roseus hairy roots were homogenized with a mixture of methanol and water and filtrated. To the filtrate 10% sulfuric acid was added, followed by extraction with ethyl acetate (alkaloids are in the ionic form) to remove ballast. After centrifugation, the organic phase was discarded, the aqueous phase was treated with 10 M NaOH (to suppress dissociation of alkaloids), and the alkaloids were extracted with ethyl acetate. The organic phases were collected and evaporated to dryness [63].

Indole alkaloids from cell cultures were extracted with methanol [10,53,55]. The residues were dissolved in water and then quickly extracted with ethyl acetate phase three times after the pH of the aqueous solution was adjusted to 10 [55], or they were dissolved in HCl aqueous solution and extracted with dichloromethane at pH 11 (adjusted with 2 M NaOH) [53]. Dichloromethane was used to extract terpenoid alkaloids from a *Catharanthus roseus* cell culture [54].

Sometimes plant materials were macerated before the extraction procedure. For example, *Chimonanthus praecox* plant material was macerated with methanol four times [37]. In addition, plant materials were sometimes defatted before extraction [76].

29.4 SAMPLE PURIFICATION AND CONCENTRATION

Purification of alkaloids contained in crude plant extracts can often be achieved by reextraction of the alkaloids from the aqueous fraction with an organic solvent after the addition of alkali [3]. The crude extracts were adjusted to a high pH (usually 8.5, 9, 9.5, or 10) with aqueous ammonia [3,11,30,31,40,46,67], sodium hydroxide [2, 12], or sodium carbamate [37,69]. Then the alkaloid fraction was reextracted with chloroform [11,24,26,31,37,40,46,67–69,81,82], ethyl acetate [30,74,83], or dichloromethane [3].

Other procedures for purification of extracts were also applied. For example, the crude extract can be dissolved in methanol, ultrasonificated, and diluted by water [24]. The crude extract was

dissolved in dilute HCl, ultrasonificated, and extracted with ethylacetate and *n*-hexane; the organic layer was then discarded to remove ballast substances. The water layer was alkalized with dilute ammonia, reextracted with dichloromethane, dried, and dissolved in 20% methanol. The fraction of alkaloids (in another procedure) was dissolved in methanol, ultrasonificated, mixed with water, and extracted with hexane.

In some cases solid-phase extraction (SPE) has been used for sample preparation. For example, the crude extract from *Nauclea pobeguinii* was dissolved in methanol, ultrasonificated, diluted with water, put on to a SPE C18 cartridge, washed with 30% methanol, and eluted with 60% methanol [24]. SPE has also been applied for preliminary fractionation of an extract [28,29]. Preliminary fractionation of plant extracts was also performed by preparative thin-layer chromatography (TLC) methods [70]. This was also used to purify indole alkaloid extracts before HPLC analysis [80]. Sometimes, alkaloid extracts were concentrated using a rotary evaporator [48]. But crude extracts were also often only filtered through a filter (0.2–45 μm) [14,22,31,40,46,60], centrifuged [10,71], or filtered and centrifuged [12].

29.5 HPLC ANALYSIS OF INDOLE ALKALOIDS

Different chromatographic methods have been applied to analyze indole alkaloids. A normal-phase system has rarely been used for HPLC analysis of the group of alkaloids. The most attractive advantage of reversed-phase chromatography is the ability to determine simultaneously a wide variety of compounds that differ markedly in molecular structure, molecular mass, polarity, and acidity/basicity. The basic properties of alkaloids, which lead to a high polarity when dissolved in water, result in a decreased affinity for the lipophilic stationary phases applied. Alkaloids appear in aqueous solutions as ionized and nonionized forms, and they are difficult to separate chromatographically. The mobile-phase pH is a major factor for the separation of analytes with acid–base properties [89–93]. Retention models of ionizable solutes in liquid chromatography as a function of pH and solvent composition were reviewed by Schoenmakers and coworkers [94,95].

Silica is the most widely used material in chromatography. Silica supports are still superior to other supports in terms of efficiency, rigidity, and performance. However, there are several problems with silica-based materials: peak tailing (especially in the chromatography of basic compounds), irreproducibility for columns with the same chemistry, and limited pH stability. Protonated basic compounds can interact with residual silanol groups of the stationary phase, as shown in the equation:

$$XH^+ + SiO^-Na^+ \Leftrightarrow Na^+ + SiO^-XH^+. \quad (1)$$

Thus, in addition to the reversed-phase retention mechanism, an ion-exchange retention mechanism also occurs, which often results in asymmetry of peaks, irreproducible retention, and worse separation. The silanol interactions can be reduced by using a mobile phase with a buffer at a low pH, suppressing the silanol ionization , or a mobile phase with buffers at a high pH to suppress solute ionization. In the analysis of basic compounds, anionic ion-pairing reagents making neutral associates are employed [96,97]. Good peak symmetry and system efficiency for analysis of basic compounds was also obtained in systems containing organic amines as silanol blockers [98]. Table 29.2 presents a comparison of different chromatographic systems applied to the analysis of indole alkaloids.

29.5.1 Normal-Phase System

Normal-phase HPLC is rarely applied in separation of indole alkaloids. However, the use of these systems to analyze alkaloids is sometimes reported. Usually a silica column, a strongly polar modifier (acetonitrile), and a medium-polar diluent (dichloromethane) are used for this purpose [71].

TABLE 29.2
Systems Used for HPLC of Indole Alkaloids

Alkaloids	Source	Stationary Phase	Eluent	Detection	Remarks	Ref.
Ajmalicine, serpentine, catharanthine	*Catharanthus roseus*	Nucleosil 5 C18	MeOH–MeCN–NH_3–TEA	UV 280 nm	Monitoring of indole alkaloid production in cell culture	[52]
Ajmalicine, serpentine, catharanthine, vincristine, vindoline, vinblastine, tabersonine, secologanine	*Catharanthus roseus*	Zorbax Eclipse XDB-C18	I. MeCN– phosphate buffer at pH 6 (gradient elution) II. MeCN– phosphate buffer at pH 2	UV 220–280 nm	Screening of the indole alkaloids of *Catharanthus roseus* hairy roots	[14]
Sellowine, vomilenine, perakine, raucaffrinoline, picrinine, epoxykuammicine	*Rauvolfia sellowii*	Lichrosorb ODS	MeCN–H_2O–NH_3	UV 222 nm	Qualitative analysis	[18]
Monoterpene indole alkaloids	*Psychotria suterella* Mull. Arg.	SiO_2	$CHCl_3$–MeOH	—	Semipreparative isolation	[83]
Ergot alkaloids	*Lolium perene*, *Neotyphodium lolii*	Luna C18	MeCN–H_2O–HCOOH (gradient elution)	200–600 nm	Qualitative analysis	[46]
Mosachamine, centcyamine	*Centaurea cyanus*	C18	MeOH–H_2O	—	Preparative isolation	[48]
Infractine	*Cortinarius infractus*	Discovery ZR-PS	MeOH–phosphate buffer at pH 2.5–NH_3	MS/MS	Qualitative analysis	[75]
Indole alkaloids	*Glycosmis montana*	XTERRA C18	MeOH–H_2O	UV, NMR	Qualitative analysis	[15]
Monoterpenoid indole alkaloids	*Psychotria leiocarpa*	C18	MeOH–H_2O (gradient elution)	UV, NMR	—	[78]
Alstilobianes	*Alstonia angustiloba*	SiO_2 ODS	$CHCl_3$–MeOH MeOH–H_2O	UV 210 nm	Purification fractions	[11]
Catharanthine, vindoline	*Catharanthus roseus*	C18	MeCN–H_2O–TFA	UV	Quantitative analysis	[7]
Brachycerine, umbellatine, lyaloside, myrianthosines, vincosamide	*Psychotria*	C18	MeOH–H_2O	UV 100–400 nm	Qualitative analysis	[79]
Macrolactam-type indole alkaloids	*Ipomoea obscura*	C-2/C18	MeOH–H_2O	UV	Isolation	[76]

Monoterpenoid indole alkaloids	*Ophiorrhiza pumila*, *Camptotheca acuminata*, *Nothapodytes foetida*	C18	MeOH–H_2O–CH_3COOH	UV	Separation and identification	[60]
Cntcyamine, moschamine, montamine	*Centaurea montana*	C18	MeCN–H_2O	UV	Isolation	[28]
Schischkiniins	*Centaurea schischkinii*	C18	MeOH–H_2O	UV	Isolation and purification	[29]
Vincamine, epivincamine	—	Chiralpak AD	iPrOH–hexane EtOH–hexane	UV	Separation of isomers	[114]
Camptothecin, hydrocamptothecin, metoxycamptothecin	*Camptotheca acuminata*	ODS	MeOH–H_2O	UV	Quantitative analysis	[22]
Ajmalicine, serpentine	*Catharanthus roseus*	Luna C18	MeCN–H_2O–HCOOH	UV, NMR, MS	Quantitative analysis	[61]
Strychnine, brucine	*Strychnos nux-vomica*	NH_2	MeCN–H_2O–CH_3COOH	UV, NMR, MS	Quantitative analysis	[101]
Quaternary indole alkaloids	*Strychnos guianensis*	C8	MeOH–MeCN–KH_2PO_4–sodium laurylosulfonate	250 and 420 nm	Qualitative analysis	[68]
Ajmalicine	*Nauclea pobeguinii*	C18	MeCN–H_2O MeCN–H_2O–DEA–HCOOH	—	Quantitative analysis	[24]
Harmine, harmaline, tetrahydroharmine	*Ayahuasca*	C18	MeOH–MeCN–H_2O–ammonium acetate	UV 260, 340, 370 and 495 nm	Quantitative analysis	[44]
Ajmaline, ajmalicine, raucaffricine, serpentine, yohimbine	*Rauwolfia serpentina*, *Rauwolfia vomitoria*	C8	MeCN–H_2O–TFA	UV 280 nm	Qualitative analysis	[2]
Harmine, harmaline	*Peganum harmala*	ODS	iPrOH–MeCN–H_2O–HCOOH–TEA	UV	Quantitative analysis	[31]
bis-Indole alkaloids	*Catharanthus roseus*	C18	MeCN–H_2O–TFA	UV	Quantitative analysis	[8]
Monoterpenoid indole alkaloids	*Rauwolfia serpentina*	C18	MeC-phosphate buffer–hexanesulfonic acid (gradient elution)	MS, NMR	Qualitative analysis	[1]
β-Carboline alkaloids	—	C18	EtOH–phosphate buffer–CD MeOH–phosphate buffer–CD	UV 290 or 430 nm	Separation of isomers	[100]

(*Continued*)

TABLE 29.2 (CONTINUED)
Systems Used for HPLC of Indole Alkaloids

Alkaloids	Source	Stationary Phase	Eluent	Detection	Remarks	Ref.
Harmine, harmane, harmalol, harmaline	*Peganum harmala*	C18	iPrOH–MeCN–H_2O–HCOOH	UV 330 nm	Quantitative analysis	[67]
Moschamine, *cis*-moschamine, centycyamine, *cis*-centycyamine	*Centaurea moschata*	C18	MeOH–H_2O	NMR	Isolation on preparative scale	[87]
Ergot alkaloids	*Claviceps purpurea*, *Neotyphodium* sp.	C18	MeCN–H_2O–ammonium acetate (gradient elution)	Fluorescence detection	Qualitative analysis	[71]
Ergovaline	*Festuca arundinacea*	C18	MeOH–H_2O–NH_3 (gradient elution)	Fluorescence detection	Quantitative analysis	[46]
Strychnine	*Strychnos nux-vomica*	C18	MeCN–H_2O–ammonium acetate	MS	Analysis	[40]
Harmine, luteonins, canthin-6-one	*Peganum nigellastrum*	SiO_2	AcOEt–hexane	NMR	Isolation	[80]
Ajmaline, vomilenine, yohimbine, ajmalicine, reserpine, strictosidine	*Rauwolfia serpentina*	C18	MeCN–Na_2HPO_4–heptanesulfonic acid	UV 255 nm	Qualitative and quantitative analysis	[88]
Ergot alkaloids	*Claviceps purpurea*	C18	MeCN–H_2O–$(NH_4)_2CO_3$	UV	Qualitative analysis	[113]
Brucine	*Strychnos nux-vomica*	C18	Sodium acetate–citric acid–sodium octyl sulphate–EDTA–dibutylamine–MeOH–H_2O	Electrochemical detection	Quantitative analysis	[109]
Catharanthine, ajmalicine	*Catharanthus roseus*	C18	MeOH–MeCN–ammonium acetate–TEA	UV	Quantitative analysis	[57]
Monoerpenoid indole alkaloids	*Catharanthus roseus*	C18	MeOH–MeCN–ammonium acetate–TEA	UV	Ultra performance liquid chromatography	[63]
Camtothecin-related alkaloids	*Ophiorrhizia pumila*	SiO_2	MeOH–$CHCl_3$	UV	Purification	[99]
Pentacyclic oxindole alkaloids	*Uncaria tomentosa*	C18	MeOH–MeCN–phosphate buffer pH 6.6	UV	Qualitative analysis	[30]
Schischkiniins	*Centaurea schischkinii*	C18	MeCN–H_2O	UV	Semipreparative HPLC, quantitative analysis	[29]

Ajmalicine, catharanthine	*Catharanthus roseus*	C18	MeOH–MeCN–diammonium hydrogen phosphate	UV	Quantitative analysis	[55]
Serpentine, ajmalicine, tabersonine, horhammericine, lochnericine	*Catharanthus roseus*	C18	MeO–MeCN–diammonium hydrogen phosphate	UV	Quantitative analysis	[11]
Terpenoid indole alkaloids	*Catharanthus roseus*	C18	MeOH–MeCN–ammonium acetate–triethylamine	UV	Quantitative analysis	[58]
Serpentine, tetrahydroyochimbine	*Rauvolfia serpentina*	C18	MeCN–H_2O–TFA	UV	Qualitative analysis	[73]
Uncarine, mitraphylline	*Uncaria tomentosa, Uncaria guianensis*	C18	MeCN–H_2O–TFA	UV 280 nm	Quantitative analysis	[66]
Psilocybin, psilocin	*Psilocybe cubensis*	C18	MeCN–ammonium formate buffer pH 3.5	UV 220 nm	Quantitative analysis	[103]
Ampthothecin	*Ophiorrhiza pumila*	AQUASIL	$CHCl_3$–MeOH–H_2O	Fluorescence detection	Quantitative analysis	[65]
Vinblastine	*Catharanthus roseus*	C18	MeOH–MeCN–buffer at pH 7	UV 255 nm	Quantitative analysis	[12]

Note: MeOH, methanol; MeCN, acetonitrile; TEA, triethylamine; iPrOH, isopropanol; TFA, trifluoroacetic acid; MS, mass spectrometry; NMR, nuclear magnetic resonance.

Addition of water to the mobile phase has been said to improve separation of ergot alkaloids [71]. For analysis of indole alkaloids from *Haraldiophyllum* species on a silica column, a mixture of ethyl acethate and *n*-hexane was used [74], and for the analysis of alkaloids from *Psychotria suterella* a mixture of MeOH and chloroform was used as the eluent [83]. A similar eluent system was applied on a SiO_2 column for separation of camptothecin-related alkaloids from *Ophiorrhiza pumila* [99]. Indole alkaloids from *Peganum nigellastrum* plant extract were analyzed on a silica column with a mixture of chloroform and ethyl acetate [80]. The indole alkaloids from *Haraldiophyllum* species were also separated on a cyanopropyl column using a nonaqueous eluent containing 2-propanol and *n*-hexane [74].

29.5.2 Reversed-Phase System

Most HPLC procedures for separating indole alkloids use an alkylsilica-bonded stationary phase. Most often, C18 stationary phases [3,10,11,14,15,22,24,28,31,40,45,46,60,61,69,71,73,78,79,100] were used, and rarely C8 [3,45,71] or C2 [76] phases. For separation of strychnine and brucine from semen *Strychni* extract, an aminopropyl stationary phase and acetonitrile with acetate buffer as the eluent were used [101]. The separation of indole alkaloids from *Cortinarius infractus* extract was performed on a ZR-PS column containing spherical, porous zirconia particles with cross-linked polystyrene, which eliminates the interaction between alkaloid molecules and free silanol groups [75]. Camptothecin was analyzed on an AQUASIL C18 stationary phase, which combines a moderate-coverage C18 group and an additional polar group, giving new selectivity compared to a traditional C18 [99]. AQUASIL C18 columns contain a polar C18 phase that provides greater retention of polar compounds and complete stability in 100% aqueous mobile phases [65].

The retention parameters and separation selectivity can be controlled by changing the eluent composition, the modifier type and concentration, the buffers added and their pH, the ion-pair (IP) type and concentration, or the type of silanol blocker and its concentration. Additionally, the retention and selectivity can be controlled by changing the stationary phase.

29.5.2.1 Mobile Phases at Low pH

Addition of an acidic buffer or acids to the mobile phase suppresses the ionization of free silanol groups, which reduces ion exchange between free silanols and alkaloid cations. In an acidic mobile phase, more symmetrical peaks, an increase in system efficiency, and a decrease in retention were obtained. This method was applied for separation of indole alkaloids, such as ajmalicine and serpentine, from *Catharanthus roseus* [14,61]. A mobile phase at an acidic pH was also used for HPLC analysis of ergot alkaloids in *Neotypodium* species [47]. A plant extract containing monoterpene alkaloids from *Psychotria umbellata* was analyzed using a mobile phase containing added acetic acid [36]. Good separation of simple indole alkaloids from *Psilocybe cubensis* (a hallucinogenic mushroom) was obtained on a C18 column with a mobile phase containing acetonitrile and an acidic buffer at pH 3.5 [102]. Formate buffer at pH 3.5 was applied to analyze β-carboline hallucinogenic alkaloids [103]. Addition of acetic acid to the mobile phase was used for the separation of camptothecin derivatives from different plant extracts [23,60], formic acid was added for the separation of β-carboline alkaloids in *Pegnanum harmala* plant extract [67] or of hallucinogenic indole alkaloids [33,60] (Figure 29.4).

Good separation of monoterpenoid indole alkaloids was obtained on a C18 column with addition of 0.1% trifluoroacetic acid to acetonitrile–water [2,10] or methanol–water [73] mixtures used as eluents (Figure 29.5) [2]. The peaks obtained on the chromatogram were symmetric and well separated. Addition of trifluoroacetic acid to a mobile phase containing acetonitrile and water was successfully used to separate terpenoidal alkaloids from *Uncaria tomentosa* and *Uncaria guianensis* plant extracts [66]. Addition of lactic acid to a mobile phase containing a mixture of 2-propanol and water was used for separation of ergot alkaloids on a C18 column [71]. Ibogaine was determined

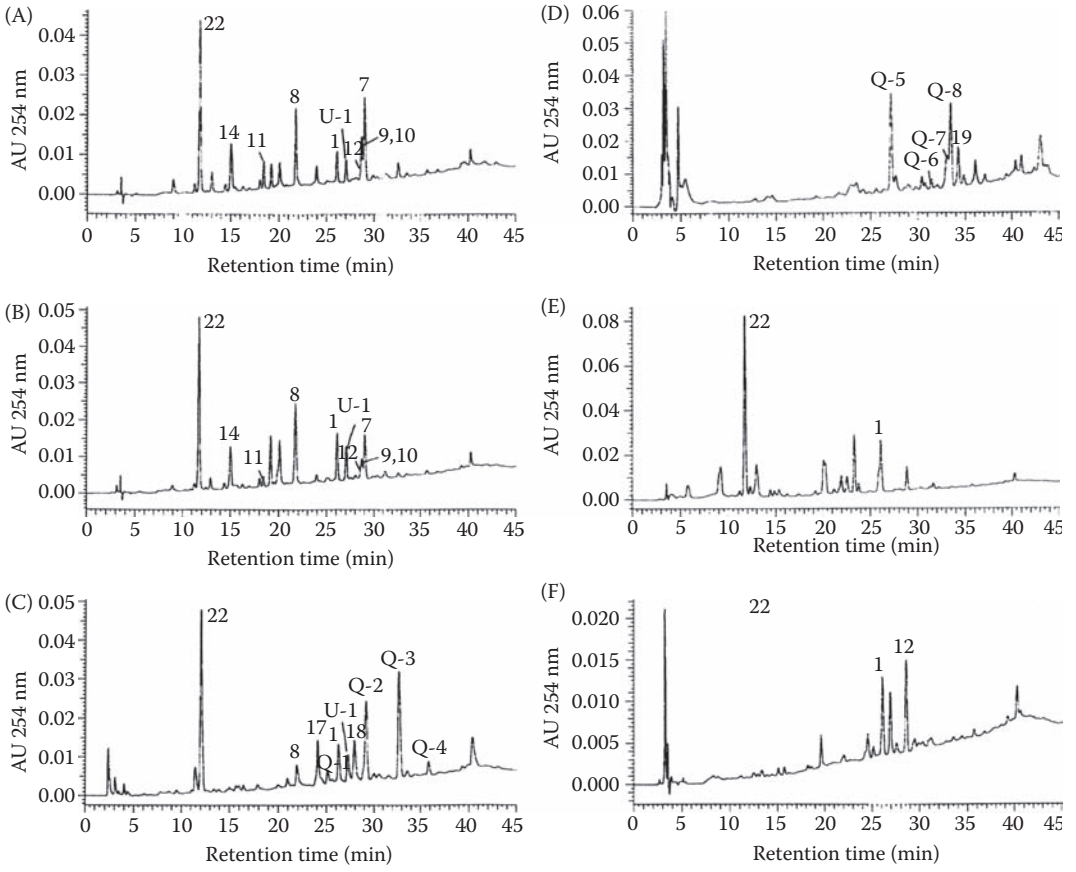


FIGURE 29.4 HPLC-diode array detection chromatograms of plant extracts detected at 254 nm. (A) *Ophiorrhiza pumila*, pot-grown plant extracts, (B) *Ophiorrhiza pumila* sterile plant extracts, (C) *Ophiorrhiza pumila* hairy root extracts, (D) *Ophiorrhiza pumila* callus extracts, (E) *Camptotheca acuminata* plant extract, (F) *Nothapodytes foetida* plant extracts. 1, Camptothecin; 7, strictosamide; 8, 3(*S*)-pumiloside; 9, 3(*S*)-deoxypumiloside; 10, 3(*R*)-deoxypumiloside; 11, chaboside; 12, 9-methoxycamptothecin; 14, strictosidinic acid; 17, lucidin 3-*O*-β-purimeveroside; 18, 3-hydroxy-2-hydroxymethylanthraquinone; 19, 1-hydroxy-2-hydroxymethyl-3-methoxyantraquinone; 22, 3-*O*-caffeoylquinic acid; U-1, Q-1–Q-8, unknown compound having an anthraquinone skeleton. The separation was carried out on a Mightysil-RP 18 column. Elution gradient program was as follows: 0–35 min, linear gradient from solvent A (water–acetic acid–methanol [79.8:0.2:20]) to solvent B (water–acetic acid–methanol [9.75:0.25:90]); 35–40 min, isocratic at 100% solvent B. (From Yamazaki, Y., Urano, A., Sudo, H., Kitajima, M., Takayama, H., Yamazaki, M., Aimi, N., and Saito, K., *Phytochemistry*, 62, 461–470, 2003. With permission.)

by use of an eluent system containing acetonitrile and formate buffer at pH 3 [104]. Indole alkaloids from *Himatanthus lancifolius* were well separated on a C18 column using an eluent system containing 0.5% phosphoric acid in a mixture of acetonitrile and water [69]. β-Carboline alkaloids from *Cortinarius infractus* were analyzed with a mixture of acetonitrile, water, and phosphate buffer at pH 2.5 [75]. The method causing the suppression of free silanols can be used only for separation of strongly retained alkaloids.

29.5.2.2 Mobile Phases at High pH

Silanol interaction can be reduced by using a high pH mobile phase, which suppresses ionization of alkaloids. The terpenoidal indole alkaloids were chromatographed on an octadecyl column with a mixture of acetonitrile and aqueous ammonia (pH 7.8) [3]. Ergovaline in cultivars of *Festuca*

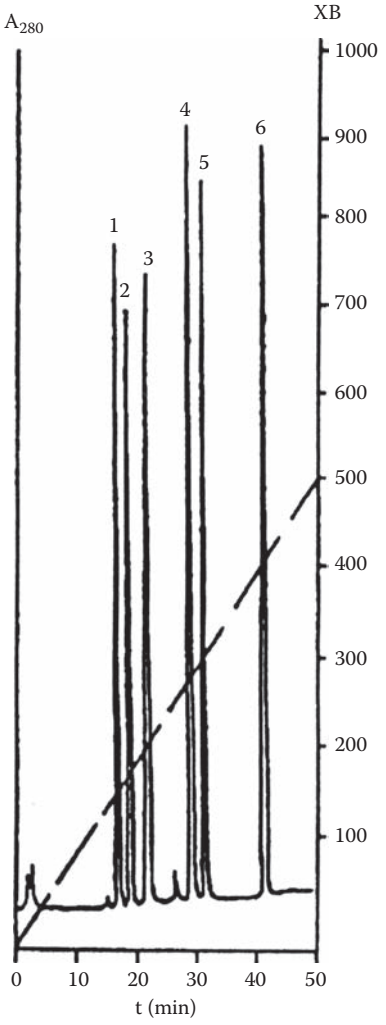


FIGURE 29.5 Separation of indole alkaloids by HPLC on an Armsorb-300-C8 column: 1, raucaffricine; 2, ajmaline; 3, yochimbine; 4, ajmalicine; 5, serpentine; and 6, reserpine. Mobile phase: (A) 10% acetonitrile and 0.1% trifluoroacetic acid in water; and (B) 0.1% trifluoroacetic acid in acetonitrile. The percentage of eluent B in the mobile phase is shown by the dashed line. (From Klyushnichenko, V. E., Yakimov, S. A., Tuzova, T. P., Syagailo, Ya. V., Kuzovkina, I. N., Wulfson, A. N., and Miroshnikov, A. I., *J. Chromatogr. A*, 704, 357–362, 1995. With permission.)

arundinacea infected by the endophyte *Neotyphodium coenophialum* was analyzed on a C18 column with a mobile phase containing methanol, water, and 0.03% ammonia [46]. Ergot alkaloids from sclerotia of *Claviceps purpurea* were chromatographed in a similar chromatographic system (C18 column with eluent containing acetonitrile and 0.01% aqueous solution of ammonia) [45]. Indole alkaloids from *Corthinarius infractus* plant extract were separated in an eluent system containing acetonitrile, phosphate buffer at pH 2.5, and 0.1 M aqueous ammonia (Figure 29.6) [75]. For separation of harmine and harmaline (β-carboline alkaloids) on a C18 column, methanol, acetonitrile, and acetate buffer at pH 8 was used as the mobile phase [44]. A mobile phase containing methanol and acetate buffer at pH 8.3 was applied to HPLC determination of the psychoactive indole alkaloid psilocin [105].

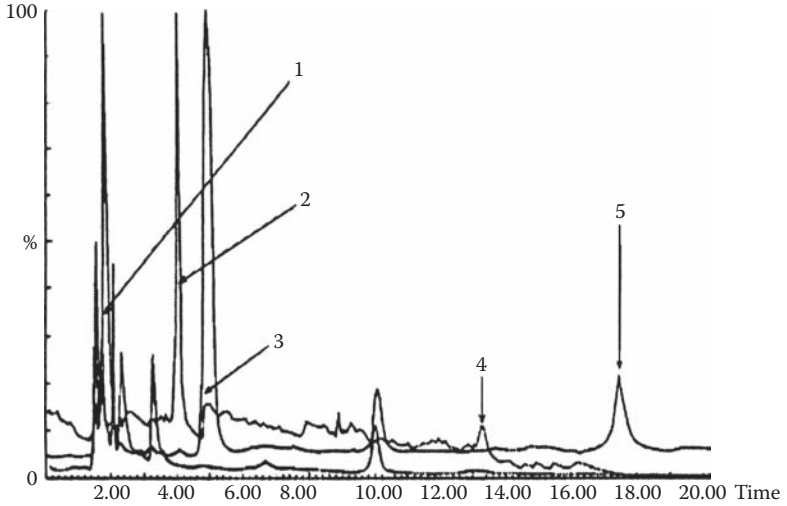


FIGURE 29.6 Total ion current recorded for HPLC–mass spectrometry analysis of extracts of *Cortinarius infractus*. The structures of substances resolved are 1, β-carboline-1-propionic acid; 2, 6-hydroxyinfractine; 3, an ethyl analogue of 6-hydroxyinfractine; 4, infractine, and 5, and ethyl analogue of infractine. The separation was carried out on a Discovery ZR-PS column. The composition of the mobile phase was acetonitrile, 0.1 M ammonium hydroxide, and water (60:20:20). (Adapted from Brondz, I., Ekeberg, D., Høiland, K., Bell, D. S., and Annino, A.R.,, I., et al., *J. Chromatogr. A*, 1148, 1–7, 2007. With permission.)

29.5.2.3 Ion-Pair (IP) Systems

A highly effective but more expensive method for avoiding the ionic mechanisms is the use of IP reagents such as alkylsulfonic acids as eluent additives [88]. Heptanesulfonic acid as an ion-pairing reagent was applied to the separation of 23 indole alkaloids from *Rauvolfia serpentina* and *Rhazya stricta* plant extracts [1,88]. Figure 29.7 presents chromatograms obtained for separation of *Rauvolfia serpentina* plant extracts in a system (a) without the addition of an ion-pairing reagent and (b) in an eluent system containing hexanesulfonic acid [1]. The next figure presents chromatograms obtained by use of a C18 column with an eluent system containing different concentrations of hexanesulfonic acid as an ion-pairing reagent. The influence of the ion-pairing reagent or buffer concentration and the pH of the mobile phase was examined. For separationof quaternary indole alkaloids from *Strychnos guianensis* plant extract, sodium laurylosulfonate was applied as an ion-pairing reagent [68].

29.5.2.4 Mobile Phases with Addition of Silanol Blockers

Systems containing amines in eluents, which play the role of silanol blockers, were more effective, than systems containing ion-pairing reagents. Thus improvement of peak symmetry and efficiency is noticed, with narrow and very symmetric peaks. The more basic compound will interact more strongly with residual silanols, allowing the less basic compound to interact solely with the alkyl ligand of the stationary phase. Indole alkaloids in different plant extracts were analysed be addition to mobile phase silanol blockers [16, 24, 31, 52, 55, 63, 106–109]. The addition of silanol blockers to mobile phase causes considerable improvement of peaks' shape, the increase of system efficiency and improvement of separation selectivity.

For analysis of indole alkaloids in a reversed-phase system, mobile phases containing only organic modifier and water [29,37,64,77,79,82,87,110,111], or organic modifier and buffer at pH 7, have often been applied [11,70,112]. In systems containing an organic modifier and water, tailing peaks and low efficiency were found. Under these conditions, alkaloids are present as ionized

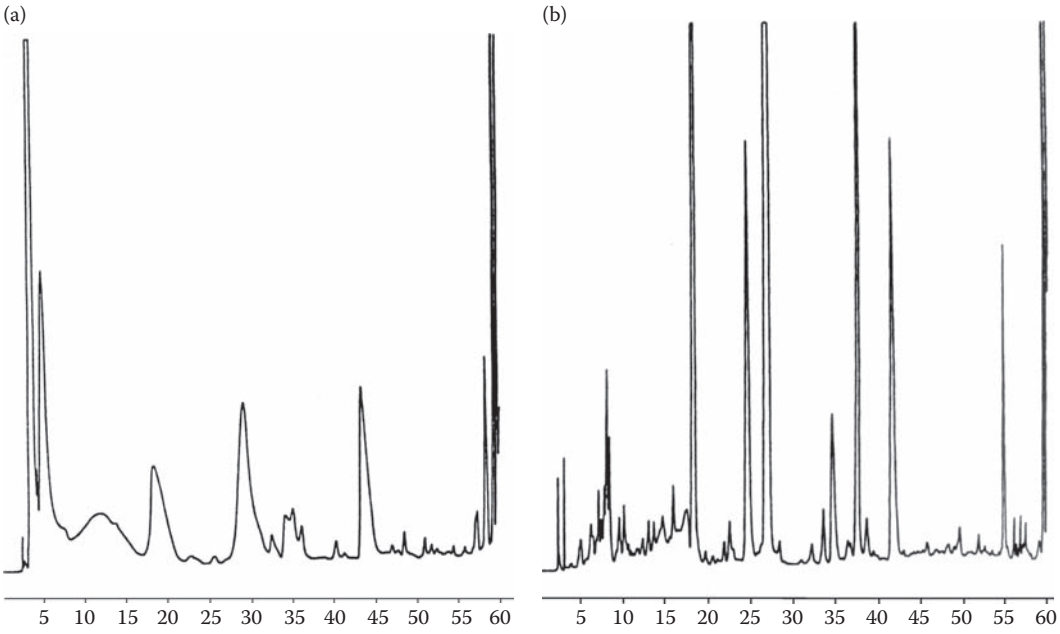


FIGURE 29.7 HPLC separation of the extracts of *Rauvolfia serpentina* X *Rauvolfia stricta* hybrid cell cultures (R X R17M). The separation was carried out on a Nucleosil 100-5 C18 column. Eluent: (A) acetonitrile, 3.9 mM dilydrogen sodium phosphate, and 1.25 mM hexanesulfonic acid; (B) acetonitrile, 3.9 mM sodium phosphate, and 2.5 mM hexanesulfonic acid. Gradient from 15:85 to 20:80 within 5 min, to 25:75 within 20 min, to 40:60 within 20 min, to 80:20 within 15 min. (From Stöckigt, J., Sheludko, Y., Unger, M., Gerasimenko, I., Warzecha, H., and Stöckigt, D., J., et al., *J. Chromatogr. A*, 967, 85–113, 2002. With permission.)

and neutral forms. Silanol groups are dissociated too. Ionized forms of alkaloids strongly interact with free silanols, which causes peak tailing, and low column efficiency. Figure 29.8 presents a chromatogram obtained for *Oxalis tuberosa* plant extract on a C18 column using multistep gradient elution (5–100% MeOH in water) [111]. For separation of alkaloids from *Uncaria tomentosa* plant extract, a mixture containing methanol, acetonitrile, and phosphate buffer at pH 6.6 was used [30]. A mobile phase at a similar pH (6.5) was used for separation of the β-carboline alkaloids harmine and harmane [40]. The monoterpenoid glucoindole alkaloids from *Palicourea adusta* extract were analyzed in a reversed-phase system with a mixture of acetonitrile, formic acid, and aqueous ammonia. For analysis of ergot alkaloids, a mixture of acetonitrile and 10 mM [113] or 2.5 mM [45] of $(NH_4)_2CO_3$ solution was applied. Good separation of psychoactive alkaloids was obtained on a C18 column with an eluent containing acetonitrile, methanol, and ammonium formate [103].

29.5.3 Chiral Separations

Recent years have seen a rediscovery of the significance of stereochemistry in drug action. There may be large differences between the receptor affinities of stereoisomers of chiral drugs, at least when the chiral center of the drug is important in its interaction with its target. For example (+)-vincamine exhibits a valuable therapeutic activity in cerebral insufficiencies. For separation of enantiomers of vincamine alkaloids, silica coated with amylose tris-3,5-dimethylphenylcarbamate (Chiralpak AD), cellulose tris-3,5-dimethylphenylcarbamate (Chiralcel OD), or (3*S*,4*R*)-Whelk O-1 [4-(3,5-dinitrobenzamido)-tetrahydrophenantrene] covalently bonded to 3-propyl silica was applied [114]. Considering that great attention has to be given to enantiomeric purity during the development

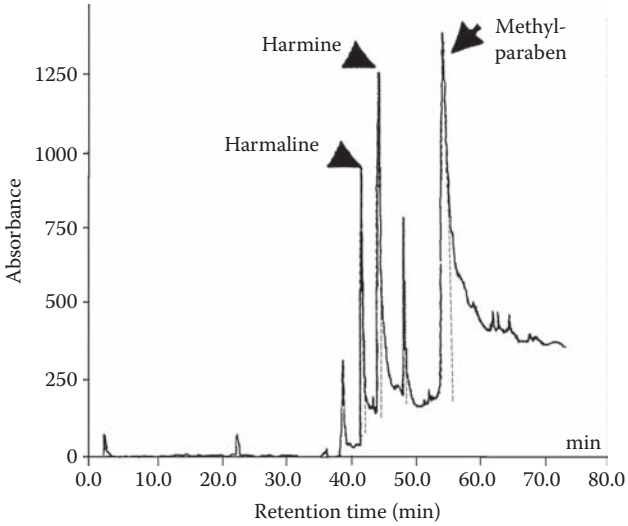


FIGURE 29.8 HPLC chromatogram of harmine, harmaline, and methylparaben from root exudates of *Oxalis tuberosa* on a Supelco C18 column. The multistep gradient was as follows: 0–5 min, 5% MeOH (methanol); 5–10 min, 20% MeOH; 15–20 min, 20% MeOH; 20–40 min, 80%MeOH; 40–70 min, 100% MeOH; 70–80 min, 5% MeOH in water. (From Bais, H. P., Park, S.-W., Stermitz, F. R., Halligan, K.M. and Vivanco, J. M., *Phytochemistry*, 61, 539–543, 2002. With permission.)

of chiral drugs. Isomers of the indole alkaloid 9-metoxymitralactonine were analyzed on a chiral column (Chiral Cel OD) with a mixture of ethanol and *n*-hexane [86].

29.5.4 Coupled Techniques

For identification of extract components, HPLC has been coupled with mass spectrometry (MS) [40, 45,47,60,71,72,75,100,101,104] or with nuclear magnetic resonance (NMR) [18,24,48,65,73,74,76,78], 2D NMR [15,28,45,68,83], or both MS and NMR [61,105]. The quantitative analysis of a strychnine and brucine mixture was conducted using tandem mass spectrometry technique provided by the triple-quadrupole LC-MS system. The HPLC chromatogram and printouts of the MS analysis are shown in Figure 29.9 [101].

29.5.5 Quantitative Analysis

The HPLC method has often been used for quantitative determination of indole alkaloids. Catabolism of *Catharanthus roseus* alkaloids was quantified during the drying process of plant material. Quantitative analysis by HPLC was used for determination of harmol, harmine, and harmaline in *Peganum harmala* plant extract [31]. The analysis was performed using the external standard method on the basis of peak area in linear regression mode. Excellent linearity for the alkaloids was obtained in the range 9.375–500 μg/mL, a correlation coefficient from 0.9997 to 0.9999. HPLC was also used for quantification of ajmalicine in extract from *Nauclea pobeguinii* [24], three indole alkaloids from *Camptotheca acuminata,* schischkiniins from *Centaurea schischkinii* [29], dimeric indole alkaloids from *Centaurea montana* [28], oxindole alkaloids from *Uncaria tomentosa* [30,66], and indole alkaloids contained in *Catharanthus roseus* plant extract [14].

The terpenoid indole alkaloid content of different plant organs of *Catharanthus pusillus* was investigated during the development from young to old plants by HPLC [85]. HPLC was also used to quantify the accumulation of indole alkaloids in a *Catharanthus roseus* cell culture [115].

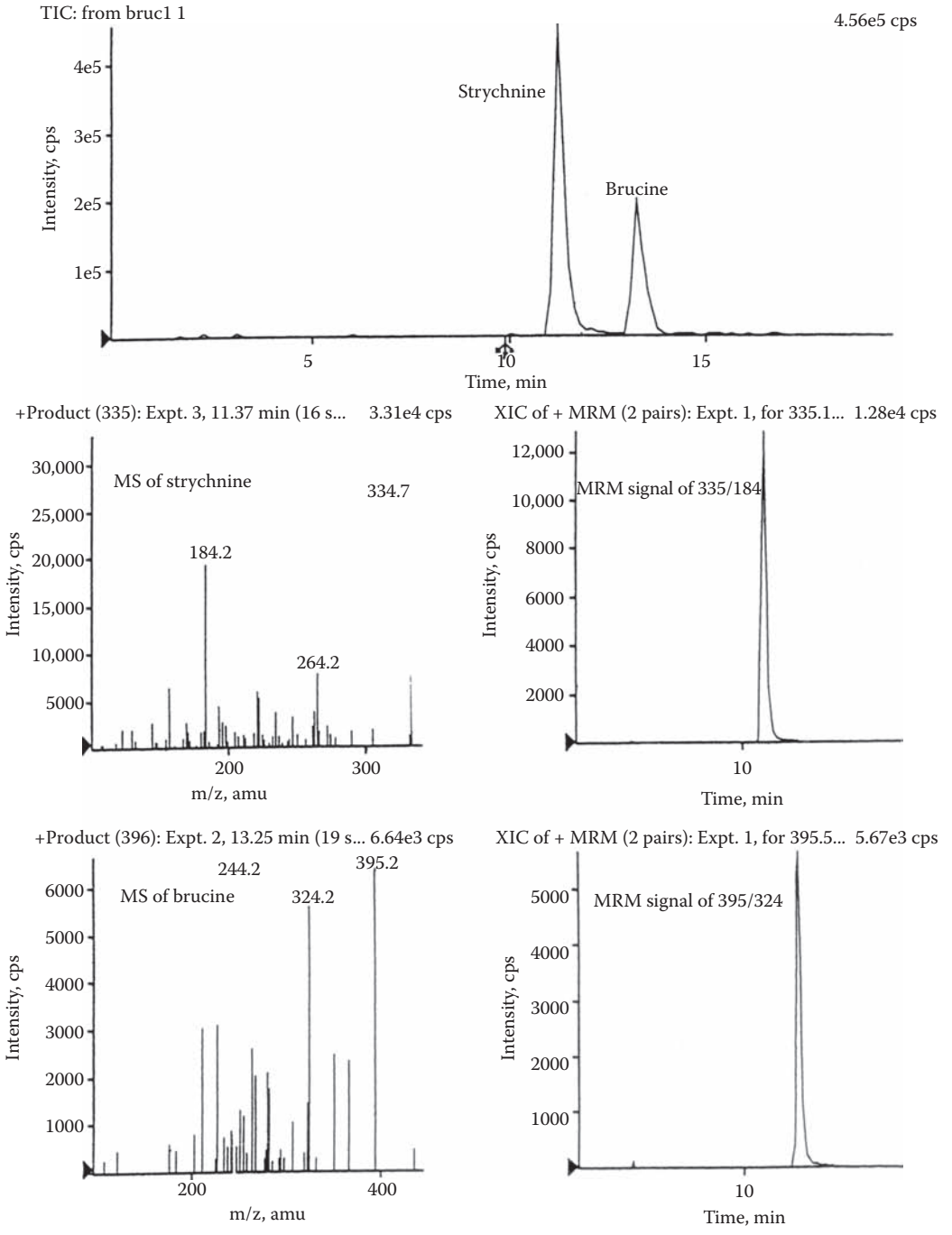


FIGURE 29.9 Chromatogram and printout of the mass spectrometric analysis of strychnine and brucine from Semen Strychni. The alkaloids were separated on an amino column with eluent containing acetonitrile, 0.4% acetic acid, and 10 mM ammonium acetate in water. (From Wong, S., Tsui S., and Kwan, S., *J. Pharm. Biomed. Anal*., 30, 161–170, 2002. With permission.)

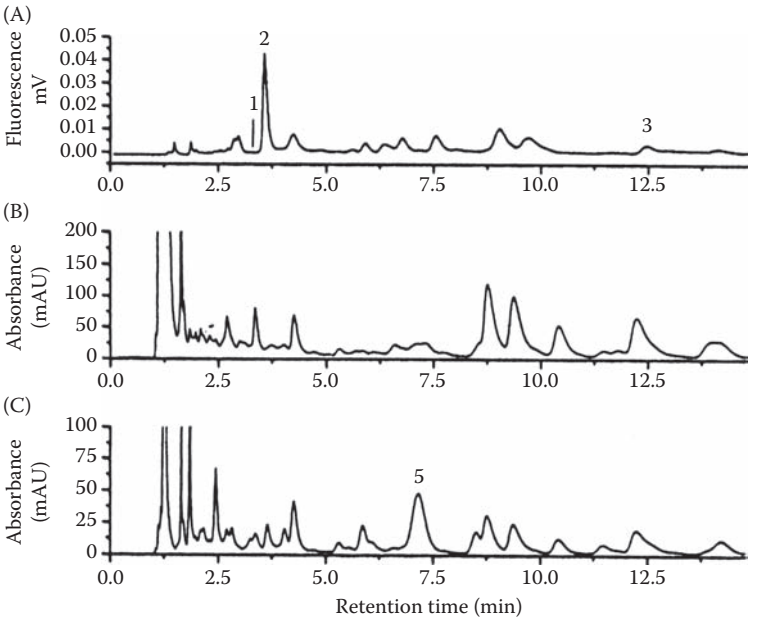


FIGURE 29.10 HPLC chromatograms of a *Catharanthus roseus* hairy root extract obtained on an XDB-C18 column. The mobile phase contained acetonitrile and phosphate buffer at pH 2 (15:85). 1, Tryptophan; 2, tryptamine; 3, ajmalicine; 4, unknown; 5, secologanine. (A) Fluorescence response at 370 nm for an excitation wavelength of 270 nm; (B) UV absorbance at 210 nm; and (C) UV absorbance at 238 nm. (From Tikhomiroff, C., and Jolicoeur, M., *J. Chromatogr.*, 955, 87–93, 2002. With permission.)

29.6 DETAILS OF SEPARATIONS OF PARTICULAR TYPES OF INDOLE ALKALOIDS

29.6.1 Simple Indoles (β-Carboline Alkaloids)

β-Carboline alkaloids (e.g., harmine, harmaline, and tetrahydroharmine) can stimulate the central nervous system by inhibiting the metabolism of amine neurotransmitters or by direct interaction with specific receptors. They are found in numerous plants, including *Pegnanum harmala*, *Passiflora incarnata*, and *Banisteriopsis caapi*, and in the entheogen preparation Ayahuasca, which is traditionally brewed using *Banisteriopsis caapi* to enhance the activity of amine hallucinogenic drugs [116]. For the analysis of β-carboline alkaloids, gradient elution on a C18 column was used. A mixture of acetonitrile, methanol, and ammonium acetate buffer at pH 8 was applied. The fluorescence detector was operated at the following excitation/emission wavelengths: 260/370 nm or 340/495 nm [44]. β-Carboline alkaloids were determined on a C18 column also with a mobile phase at an acidic pH. For example, alkaloids in seed extract of *Peganum harmala* were chromatographed with a mixture containing 2-propanol, acetonitrile, water, and formic acid as the mobile phase [67]. Psilocin and psilocybin from *Psilocybe cubensis* were separated on a C18 column at an acidic pH (mobile phase containing acetonitrile and ammonium formate buffer at pH 3.5) [102]. Extract from *Cortinarius infractus* containing β-carboline alkaloids (infractines) was analyzed on a Zr-PS stationary phase comprised of spherical zirconia particles with cross-linked polystyrene with an acidic aqueous mobile phase (pH 2.5) [75]. Psilocybin, the main psychoactive compound of *Psilocybe* mushrooms, was analyzed on a C18 column with a mobile phase containing acetate buffer at pH 8.3 [105]. The major simple alkaloids such as harman, harmine, and harmaline are found in *Passiflora* and *Peganum* species and the Elaegnaceae family. Separation was achieved using a C18 column and mixture of isopropyl alcohol, acetonitrile, water, formic acid, and triethylamine as the eluent (Figure 29.11) [31].

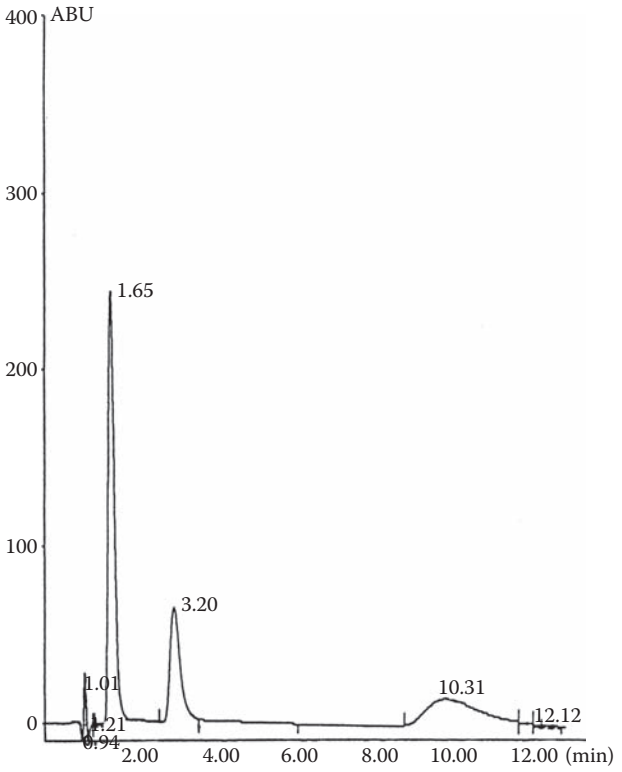


FIGURE 29.11 Chromatogram of the Semen Pegani extract obtained on a Metasil ODS column. The mobile phase contained isopropyl alcohol, acetonitrile, water, and formic acid (100:100:300:0.3). 1.65 harmol; 3.20 harmine; 10.31 harmaline. (From Kartal, M., Altum, M. L., and Kurucu, A. S., *J. Pharm. Biomed. Anal.*, 31, 263–269, 2003. With permission.)

β-Carboline alkaloids were chromatographed on a C18 phase with addition of methyl cyclodextrins to the mobile phase [100]. The presence of cyclodextrins in the mobile phase alters the chromatographic equilibria and induces a secondary chemical equilibrium associated with the chromatographic separation by HPLC. Cyclodextrins as well as other macrocyclic compounds present peculiar host–quest interactions, recognizing and differentiating analytes on the basis of their size, shape, polarity, and polarizability. Consequently, cyclodextrins have been employed to enhance the HPLC separation of chemically related compounds, such as the indole alkaloids norhamane, harmane and harmine, which differ in a methyl group at the C-1 position and a metoxy group at the C-7 position on the β-carboline ring. These alkaloids were well separated on a C18 stationary phase with an eluent containing methanol or ethanol as an organic modifier, phosphate buffer at pH 7.8, cyclodextrin, and *tert*-butyl alcohol. *Tert*-butyl alcohol in the mobile phases can contribute to the stabilization of the inclusion cyclodextrin complexes.

29.6.2 Ergot Alkaloids

One of the pharmacologically most important groups of indole alkaloids are ergot alkaloids. These alkaloids are isolated from the dried sclerotium of the fungus *Claviceps purpurea* (ergot). This fungus is a parasite on rye and wheat grains. Numerous semisynthetic derivatives of the ergot alkaloids have been prepared, and several are of therapeutic interest. Ergot alkaloids have often been analyzed by HPLC on a C18 column with an acidic mobile phase (with addition of formic acid)

[6,47]. Ergovaline and ergine were separated by HPLC on a C18 column with a mobile phase containing 2-propanol, water, and 1% lactic acid. The mixture of acetonitrile and aqueous ammonium acetate was also applied as a mobile phase to the separation of ergot alkaloids [71]. Ergot alkaloids were analyzed in system containing acetonitrile and a solution of $(NH_4)_2CO_3$ [45,113]. Eluent systems at a basic pH were also used. A mobile phase containing a mixture of methanol and water with addition of 0.03% aqueous ammonia was applied to determination of ergovaline in extract from cultivars of infected *Festuca arundinacea* [46], and an eluent containing 0.01% ammonia was used for separation of ergot alkaloids from *Claviceps purpurea* on a C18 or C8 stationary phases (Figure 29.12) [45].

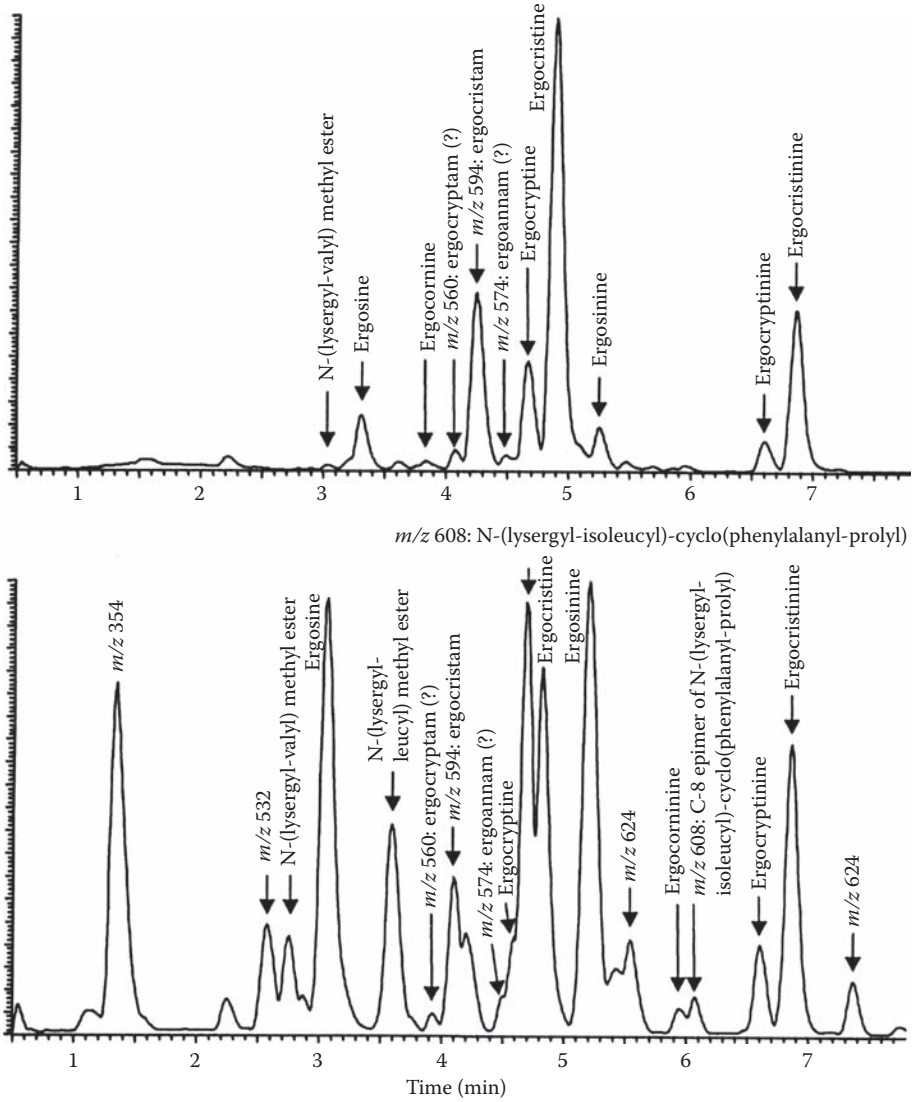


FIGURE 29.12 Chromatograms from HPLC–electrospray ionization (positive mode)–mass spectrometry of the methanolic extract from *Claviceps purpurea* sclerotia. (A) Sclerotia from *Phelum pretense*; (B) sclerotia from *Molinia caerulea*. The alkaloids were separated on a SunFire C8 column. The mobile phase contained acetonitrile and 0.01% ammonia in water (25:75). (From Uhling, S., and Petersen, D., *Toxicon*, 52, 175–185, 2008. With permission.)

29.6.3 Terpenoidal Indole Alkaloids

This group of alkaloids is very important because of their actual and potential usefulness as pharmaceuticals. In recent years, there have been significant developments in biotechnological methods for obtaining indole alkaloids, apart from their isolation from natural plants. An interesting technique for the synthesis of indole alkaloids by cell culture, namely, hairy root cultures of a different plant, was described recently [22,61].

Mobile phases containing only an organic modifier and water were applied in a reversed-phase system for separation of the indole alkloids. Terpenoidal alkaloids from *Psychotria* species were analyzed on a C18 column with a linear gradient solvent system from 50% methanol in water to 100% methanol [79]. Monomeric terpenoidal alkaloids from *Psychotria leiocarpa* were separated on a C18 column with an eluent system containing only methanol and water [78]. Dimeric indole alkaloids from *Centaurea montana* plant extract were also chromatographed on a C18 column with an eluent containing only acetonitrile and water [28]. The dimeric indole alkaloids from *Catharanthus roseus*—vinblastine and vincristine, widely used in treatment of various human cancers—were analyzed on a C18 column. A mixture of methanol, acetonitrile, and buffer at pH 7 was applied as the mobile phase [11]. Pentacyclic oxindole alkaloids from *Uncaria tomentosa* were determined on a C18 column using acetonitrile with buffer at pH 7 [70]. AN HPLC fingerprint chromatogram obtained for *Uncaria tomentosa* plant extract is shown in Figure 29.13 [30].

A mixture of methanol, acetonitrile, and diammonium hydrogen phosphate was used as the eluent on a C18 column to separate indole alkaloids from *Catharanthus roseus* plant extract [53]. The separation of vincamine from vincaminic acid was achieved on a C18 column with a mobile phase consisting of acetonitrile and 0.01 M aqueous ammonium carbamate [17]. A mobile phase at an acidic pH was also applied to separate these groups of alkaloids (e.g., acetonitrile and formic acid [61], acetonitrile and phosphate buffer at pH 2 [14], or mixture of methanol and water containing acetic acid [36,60]). Terpenoidal alkaloids from *Catharanthus roseus* were analyed in a system containing a C18 stationary phase and a mixture of acetonitrile, 2-methoxyethanol, and phosphate buffer at pH 3.9 [54]. Good separation of the oxindole alkaloids speciophlline, mitraphylline, uncarine, and isomitraphylline from *Uncaria tomentosa* and *Uncaria guianensis* plant extracts was obtained with a C18 stationary phase and a mobile phase at an acidic pH [66].

An acidic mobile phase was also applied to separation of terpenoidal alkaloids from *Rauvolfia serpentina* [73]. Often, for the analysis of this group of indole alkaloids, eluents containing different amines have been used. The monomeric terpenoidal alkaloids catharanthine and vindoline were analyzed on a C18 column with acetonitrile, water, and trifluoroacetic acid as the mobile phase [10].

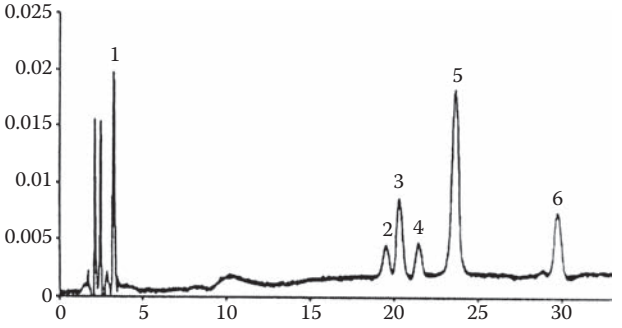


FIGURE 29.13 HPLC chromatogram of *Uncaria tomentosa* plant extract obtained on a LiChrospher 100 RP-18 column. Solvents: (A) phosphate buffer solution (10 mM, pH 6.6), (B) methanol–acetonitrile (1:1). Gradient: 60% A and 40% B to 30% A and 70% B; time 30 min. 1, Caffeine; 2, uncarine F; 3, speciophylline; 4, mitraphylline; 5, isomitraphylline; 6, isopteropodine (From Pilarski, R., Zielińsk, H., Ciesiołka, D., and Gulewicz, K., et al., *J. Ethnopharmacol*., 104, 18–23, 2006. With permission.)

Good separation of terpenoidal alkaloids from *Catharanthus roseus* was obtained by use of an aqueous mobile phase containing triethylamine [10,50]. Dimeric indole alkaloids from the *Rauwolfia* family were chromatographed on a C8 stationary phase with different mobile phases. The best separation was achieved with acetonitrile, water, and trifluoroacetic acid as eluent [2]. Good results were also obtained on an octadecyl stationary phase with eluent containing acetonitrile and aqueous ammonia [3]. Strictosamine from *Nauclea pobeguinii* was determined on a C18 column with addition of diethylamine to the aqueous mobile phase [24]. Separation of terpenoidal indole alkaloids contained in *Vinca minor* was performed on a C18 stationary phase with addition of diethylamine to an aqueous mobile phase [16].

29.6.4 Indoles with a Quaternary Nitrogen

The indole alkaloids include very toxic alkaloids such as strychnine and brucine, which were analyzed on a C18 column with a mobile phase containing acetonitrile and aqueous ammonium acetate [71]. The content of strychnine in *Strychnos nux-vomica* seeds was determined by HPLC–electrospray ionization (ESI)–MS method on a C18 column with a mixture of acetonitrile and aqueous solution of ammonium acetate as the mobile phase [40]. Strychnine and brucine were also successfully determined on an aminopropyl column with a mobile phase containing acetonitrile and acetate buffer [61]. Quaternary indole alkaloids from *Strychnos guianensis* were separated on a C8 stationary phase with application of the ion-pairing technique [68]. An aqueous mobile phase of acetonitrile, methanol, potassium dihydrogen phosphate, and sodium laurylsulfonate was used.

29.6.5 Other Indole Alkaloids

Physostigmine—the main alkaloid of *Physostigma venenosum*—is a carmamin acid derivative that arises biogenetically from 5-OH-tryptophane. The alkaloid was analyzed on a silica column with a mobile phase containing acetonitrile and 0.01 M sodium dihydrogen phosphate (pH 3) [117]. Camptothecin has a quinoline ring system but has been shown to arise biogenetically from an indolic precursor. It has been analyzed by HPLC using an ODS stationary phase and a mobile phase at a neutral pH (methanal–water gradient elution) [22] or an AQUASIL column with a solvent system containing chloroform–methanol–water (10:5:1) [65]. Physostigmine and its metabolite eseroline were separated on a silica column with an aqueous eluent containing acetonitrile and phosphate buffer at pH 3 [117]. Camptothecin and related compounds (lactone and carboxlate forms of camptothecin) were analyzed with application of acetonitrile, phosphate buffer, and a silanol-blocking agent, triethylamine [107]. Fluorescence detection was used for determination of campthotecin. The optimal fluorescence response for this alkaloid was observed at excitation and emission wavelengths of 360 and 440 nm [84]. Ipobscurines — macrolactam-type indole alkaloids that are theactive constituent of *Ipomoea obscura* — were well separated on a silica column with Me_2CO–cyclohexane [76].

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30 HPLC of Isoquinoline Alkaloids

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CONTENTS

Isoquinoline alkaloids, biogenetically derived from tyrosine, represent a manifold class of compound within the plant kingdom. Opium alkaloids are medicinally and economically by far the most important alkaloids from this group. Therefore, their analysis using high performance liquid chromatography (HPLC) will be discussed separately in the subchapter Opium Alkaloids. HPLC methods for the other alkaloids derived from tetrahydroprotoberberine will be reviewed in the subchapter Protoberberine Alkaloids and Derivatives.

30.1 OPIUM ALKALOIDS

Opium alkaloids, which play a major role in medicine and pharmacy, are biosynthesized by the opium poppy *Papaver somniferum* L. [1,2]. Opium, the dried latex collected from the unripe capsule after surface incision, is the source for the commercial production of these tetrahydrobenzylisoquinoline-derived alkaloids, including morphine, codeine, thebaine noscapine, and papaverine (Figure 30.1). Morphine is used in therapy as an analgesic for the treatment of terminal cancer patients. Codeine and noscapine are commonly used as antitussives. Papaverine serves as a muscle relaxant. Thebain is worked up by the pharmaceutical industry to produce semisynthetic derivatives such as the analgesic oxicodone and the opiate antagonists naloxone and naltrexone. Opium alkaloids including morphine, codeine, and thebaine are narcotic/addictive drugs, and their production is strictly regulated worldwide by the International Narcotic Control Board.

Due to their extensive use in medicine, hundreds of tons of opium alkaloids and semisynthetic derivatives are produced annually. Therefore, HPLC methods to determine *P. somniferum* alkaloids are required for samples such as raw plant material to evaluate alkaloid levels in different capsules, and in industrial process streams to optimize extraction yields. These methods will be discussed here. Several HPLC methods from the literature are proposed to quantify opium alkaloids in pharmaceutical formulations, and also in body fluids/tissues and hair samples for forensic analysis.

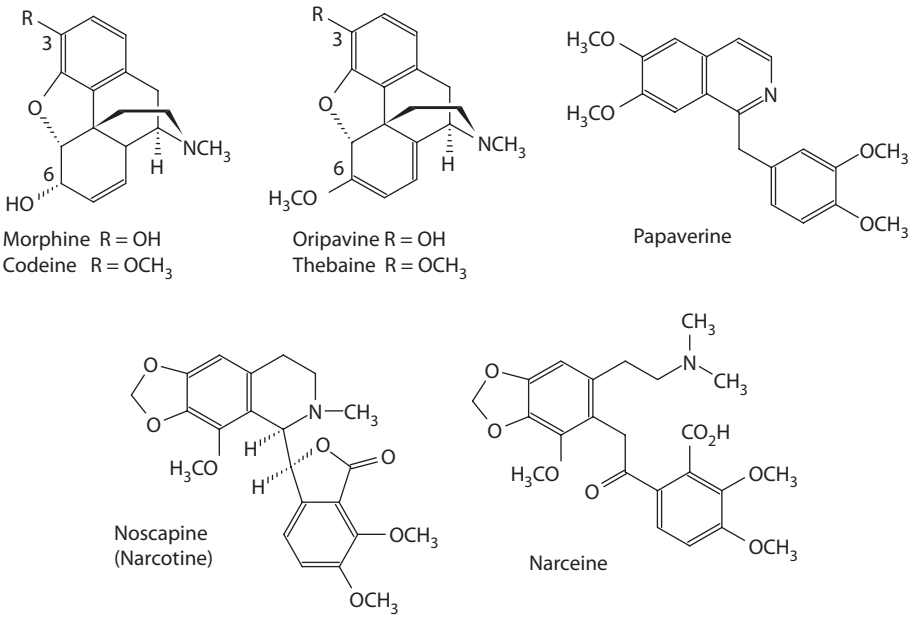

FIGURE 30.1 Chemical structures of alkaloids from *Papaver somniferum* L.

These methods are not the subject of this section. General methodology for determination of *P. somniferum* alkaloids has been discussed in previous reviews [3–7].

30.1.1 Reversed-Phase and Ion-Pair HPLC

A reversed-phase HPLC (RP-HPLC) method on a μBondapack C_{18} column has been described [8] for determination of morphine and codeine in poppy straw concentrate (Table 30.1). Separation of five major opium alkaloids (morphine, codeine, papaverine, thebaine, and noscapine) and ethylmorphine was achieved within 26 min using 0.05 M sodium acetate buffer (pH 3.6)–acetonitrile (ACN)–tetrahydrofuran (THF)–ethanol (86:6:3:3, v/v) as a mobile phase at a flow rate of 1.5 mL min^{-1}. Detection was done at 284 nm.

An isocratic RP-HPLC method was proposed by Ayyangar and Bhide [9] for the analysis of the principal alkaloids in aqueous acetic acid (2.5%) extract of gum opium (Table 30.1). Simultaneous separation of morphine, codeine, papaverine, thebaine, and noscapine was achieved within 30 min on a μBondapak phenyl column (300 × 3.9 mm ID) with methanol (MeOH) and water (58:42, v/v) supplemented with 1% sodium acetate and 7.0 mM triethylamine (TEA; pH 11) as a mobile phase. Though pH-dependent column deterioration was not observed over a period of three months without a guard column, the use of an appropriate one was recommended to prolong column life.

A gradient RP-HPLC method was developed by Ayyangar and Bhide [10] for separation of five principal alkaloids (morphine, codeine, thebaine, noscapine, and papaverine), three minor alkaloids (laudanosien, cryptopine, and narceine), and meconic acid in gum opium samples (Table 30.1). Alkaloids were extracted with 2.5% aqueous acetic acid and separated on an MN-Nucleosil 7 C_6H_5 (7 μm; 250 × 4 mm ID) phenyl column with a mobile phase consisting of triethylammonium phosphate buffer (~0.007 M; pH 2.2) and MeOH in a gradient from 5 to 70% during 20 min at a flow rate of 1.5 mL min^{-1}. A phenyl column was selected, because, as previously observed [9,11], a bonded phenyl stationary phase gave better peak shapes for alkaloids than an octadecyl phase under comparable conditions. The five principal alkaloids were quantified at 280 nm with good precision.

TABLE 30.1
HPLC Methods for Analysis of Opium Alkaloids

Alkaloids	Aims	Stationary Phase	Column Dimensions L x ID (mm)	Mobile Phase	Ref.
Morphine, codeine, ethylmorphine, thebaine, noscapine, papaverine	Analysis of alkaloids in poppy straw concentrate	μBondapack C_{18}, 10 μm	300 × 4.0	0.05M sodium acetate buffer, pH3.6–ACN–THF–ethanol (88:6:3:3, v/v)	[8]
Morphine, codeine, thebaine, noscapine, papaverine	Separation of the principal alkaloids in gum opium	μBondapack Phenyl, 10 μm	300 × 4.0	(58:42, v/v) MeOH–H_2O containing 1% sodium acetate and 7.0 mM TEA, pH 11	[9]
Morphine, codeine, thebaine, noscapine, papaverine, laudanosine, cryptopine, meconic acid	Separation and determination of alkaloids in gum opium	Waters Guard-Pak pre-column with Resolve CN, 10 μm; MN-Nucleosil 7 C_6H_5, 7 μm	250 × 4.0	A: 100 mL MeOH–H_2O (5:95, v/v) + 1.0 mL triethylammonium phosphate (~0.7 M), apparent pH 3.2 B: 100 mL MeOH–H_2O (70:30, v/v) + 1.0 mL triethylammonium phosphate (~0.7 M), apparent pH 3.95 Gradient: 0–20 min, 0%–100% B; 20–32 min, 100% B; 32–40 min, 100%–0% B; 40–50 min, 100% A	[10]
Morphine, codeine, papaverine, noscapine, sanguinarine	Identification and determination of alkaloids in nontransformed and transformed shoots of *P. somniferum*	TSK gel ODS 120 Å	250 × 4.6	A: ACN B: 10 mM sodium 1-heptanesulfonate, pH 3.5 Gradient: 0–10 min, 25%–43% A; 10–15 min, 43% A; 15–22 min, 43%–50% A; 22–26 min, 50%–80% A	[12]
Morphine, codeine, thebaine, oripavine	Qualitative and quantitative analysis of alkaloids in poppy straw	C_{18}, 4 μm, in 8 NV Radial Pak cartridge	100 × 8	A: ACN B: H_2O with 0.05 M sodium salt of octanesulfonic acid, pH 3.5 Gradient: 0–10 min, 25%–30% A; 10–17 min, 30%–60% A; 17–18 min, 60%–25% A; 18–28 min, 25% A	[13]

(*Continued*)

TABLE 30.1 (CONTINUED)
HPLC Methods for Analysis of Opium Alkaloids

Alkaloids	Aims	Stationary Phase	Column Dimensions L x ID (mm)	Mobile Phase	Ref.
Morphine, codeine, thebaine, papaverine, noscapine	Determination of five major alkaloids in opium	Inosil BA C_{18}, 5 µm	250 × 4.6	A: 1-heptanesulfonic acid sodium salt (1.0 g) in H_2O (390 mL), pH 3.2 with orthophosphoric acid B: ACN Gradient: 0–9 min, 72% A; 9–17 min, 72%–63% A; 17–20 min, 63% A; 20–21 min, 63%–0% A; 21–25 min, 0% A; 25–26 min, 0%–72% A; 26–40 min, 72% A	[15]
Morphine, codeine, thebaine, papaverine, noscapine	Separation and determination of alkaloids in gum opium	Kovasil MS-C_{18}, 1.5 µm	33 × 4.6	A: 0.1 % aqueous heptafluorobutiric acid B: ACN–H_2O (60:40, v/v) with 0.8% heptafluorobutiric acid Gradient: 0–0.3 min, 15%–29% B; 0.3–1.9 min, 29%–38% B; 1.9–2.4 min, 38% B; purging and regeneration: 2.4–2.6 min, 38%–100% B; 2.6–3.4 min, 100% B; 3.4–3.6 min, 100%–15% B; 3.6–7.0 min, 15% B	[16]
Opium alkaloids: morphine, papaverine, codeine, noscapine, oripavine, sanguinarine, thebaine	Determination of alkaloids *Papaver somniferum* L., *P. setigerum*, *P. bracteatum*, *P. pseudo-orientale*	TSK gel ODS-120 Å Temperature: 40ºC	250 × 4.6	ACN–10 mM sodium 1-heptanesulfonate in H_2O, pH 3.5 with H_3PO_4 Gradient: 0–15 min, 20%–30% A; 15–20 min, 30%–40% A; 20–25 min, 40% A; 25–30 min, 40%–50% A; 30–35 min, 50%–20% A	[19]

Note: ACN, acetonitrile; MeOH, methanol; TEA, triethylamine; THF, tetrahydrofuran.

An ion-pair HPLC method was developed by Yoshimatsu and Shimomura [12] for simultaneous analysis of morphine, codeine, papaverine, and noscapine together with sanguinarine, because transformed and nontransformed shoot cultures of *P. somniferum* readily produce sanguinarine (Table 30.1). Lyophilized samples were extracted with 5% acetic acid. After chloroform washing, the aqueous phase was made alkaline with concentrated ammonium hydroxide, and alkaloids were extracted into chloroform and finally transferred into methanol. Chromatographic separations were achieved within 27 min on a TSK gel ODS 120 Å column (250 × 4.6 mm ID) at 35ºC with an acetonitrile gradient (25–80%) and 10 mM sodium 1-heptanesulfonate (pH 3.5) as the ion-pair reagent. Alkaloids were determined at 284 nm, and nontransformed shoots contained much more codeine (1310–2030 μg g^{-1} dry weight) than morphine (50–70 μg g^{-1} dry weight). No morphine could be detected by HPLC in genetically transformed shoots.

Gomez-Serranillos et al. [13] proposed an ion-pair HPLC method for determination of the main alkaloids (morphine, codeine, and thebaine), together with oripavine, in poppy straw (Table 30.1). A column consisting of a radial compression cartridge packed with C_{18} (4 μm) as the stationary phase (10 × 8 mm ID) yielded both separations with good resolution and fast elution times (< 16 min) similar to those achieved with conventional stainless steel columns [14]. The mobile phase was a gradient of ACN and 0.05 M aqueous solution of octanesulfonic acid sodium salt, pH 3.5.

Ion-pair HPLC on a base-deactivated stationary phase was applied for determination of the five major alkaloids including morphine, codeine, thebaine, papaverine, and noscapine from *P. somniferum* (Table 30.1) [15]. Alkaloids from dried and pulverized opium were extracted twice with acetic acid (2.5%, v/v), purified at pH 9.0 (concentrated ammonia) on an Extrelut column eluted with dichloromethane + isopropanol (9 + 1.60 mL), and, after evaporation to dryness, redissolved in methanol. Because in some HPLC systems the broad peak shape of late-eluting opium alkaloids can cause problems with their precise quantification, the use of a base-deactivated stationary phase seemed promising for suppression of tailing. Therefore, a Hypersil BDS C-18 column (5 μm; 250 × 4 mm ID) and Inosil BA C-18 column (5 μm; 250 × 4.6 mm ID) were used for preliminary investigations. A slightly better separation and resolution of the five alkaloids and the internal standard brucine in only 20 min was finally achieved with a 5 μm Inositol column. Gradient elution was applied with ACN and a solution of 1-heptanesulfonic acid sodium salt (1.0 g) in water (390 mL), pH adjusted to 3.2 with orthophosphoric acid, as solvents at a flow rate of 1.8 mL min^{-1}. Alkaloids were determined at 280 nm. The precision of the method ranged from 2.79% to 4.41% ($n = 20$) for the five alkaloids investigated. The method was found to be applicable to opium and poppy straw [15].

An RP-HPLC method on a nonporous stationary phase has been proposed by Krenn et al. [16] for the rapid analysis of the main opium alkaloids morphine, codeine, thebaine, papaverine, and noscapine and the internal standard brucine (Table 30.1). Alkaloids from the gum opium samples were extracted twice with acetic acid (2.5%), purified at pH 9.0 (concentrated ammonia) on an Extrelut column eluted with dichloromethane + isopropanol (9 + 1.60 mL), and, after evaporation to dryness, redissolved in the mobile phase. Excellent separation and resolution of the six alkaloids were achieved in 1.5 min on a 1.5 μm particle size Kovasil MS-C_{18} column (33 × 4. 6 mm ID) with multilinear gradient elution. The mobile phase was prepared by mixing 0.1% aqueous heptafluorobutiric acid with 60:40 (v/v) ACN–water containing 0.8% heptafluorobutiric acid. The precision of the new method was satisfactory (0.95–2.56%) and limits of detection (LODs) ranged from 0.012 to 1.26 ng for the five alkaloids investigated. Different opium samples were analyzed using the new nonporous material and the classical porous packing [15], and good correlation was found between results obtained. The new method reduced the analysis time by a factor of ten.

The combined use of four different liquid chromatographic methods for determination of alkaloid content in poppy straw samples was proposed by the Nyiredy research group [17,18] for breeding and selection of high alkaloid-producing *P. somniferum* cultivars. Multilayer overpressured-layer chromatography (MLOPLC) was used as a semiquantitative method, and normal phase high-performance thin-layer chromatography (NP-HPTLC) as the first quantitative method for preselecting samples for further analysis. Rapid RP-HPLC was performed on a Kovasil MS-C_{18}

column (1.5 μm; 33 × 4.6 mm ID) using a solvent gradient of ACN in 5 mM aqueous 1-heptanesulfonic acid sodium salt. Six alkaloids, morphine, narcotoline, codeine, thebaine, papaverine, and narcotine were separated and determined in less than 4 min. The within-day reproducibility, as RDS, was 0.2–1.6% ($n = 5$) and between-day reproducibility was 1.3–2.7%, for all the alkaloids. A confirmatory RP-HPLC method was also proposed on a Eurospere-100 C_{18} column (5 μm; 120 × 4 mm ID) thermostated at 40°C with a solvent gradient of 40:14:46:1 (v/v) ACN–MeOH–water–THF in 100:1 (v/v) water–THF. The analysis time was 15 min. The within-day reproducibility, as RDS, was 0.3–0.8% ($n = 5$) and between-day reproducibility was 0.7–2.1%, for all the alkaloids.

A new and reliable solid-phase extraction (SPE) method was developed by Yoshimatsu et al. [19] for HPLC analysis of opium alkaloids from *Papaver* plants (Table 30.1). Previously published SPE methods can be applied only for analysis of narcotic drugs in biological fluids such as urine and plasma [20,21]. Powdered plant samples (capsules or leaves) were extracted with 5% acetic acid by sonication. After centrifugation, an aliquot of the supernatant was applied on an RP cation-exchange SPE cartridge. After column washing with 0.1 M hydrochloric acid and MeOH, alkaloids were eluted with 1:19 (v/v) 28% ammonia–MeOH. SPE method recovery for morphine, codeine, oripavine, thebaine, papaverine, noscapine, and sanguinarine ranged from 99.94% to 112.18% using fortified plant samples. The method was applied for the analysis of alkaloids in *P. somniferum*, *P. bracteatum*, and *P. pseudo-orientale* plants cultivated in the field and phytotron (Table 30.1).

30.1.2 HPLC with Chemiluminescence Detection

The determination of multiple analytes in complex sample matrices most commonly involves HPLC with UV absorbance, fluorescence, electrochemical, or mass spectrometric (MS) detection. Chemiluminescence (the emission of light from a chemical reaction) is an alternative mode of detection that provides high sensitivity using relatively simple instrumentation [22,23]. Chemiluminescense has been used to determine a wide range of *P. somniferum* alkaloids [24]. Liquid-phase chemiluminescence reagents discussed in the following exhibit remarkable sensitivity and complementary selectivity for many *P. somniferum* alkaloids, as was revealed by investigations into their chemiluminescence determination using flow-injection and sequential-injection analysis. Tris(2,2'-bipyridyl)ruthenium (III) is most suitable for the detection of nonphenolic alkaloids that possess tertiary amine functionality, such as codeine, 6-methoxycodeine, noscapine, and thebaine. Acidic potassium permanganate provides the greatest LOD for phenolic morphinan species such as morphine, oripavine, the semisynthetic derivative buprenorphine, and naloxone. Sensitive detection of papaverine is possible only by combined use of permanganate and sulfite, or of manganese (IV) and formaldehyde.

A rapid and highly sensitive HPLC method using a Chromolith SpeedROD Rp-18e monolithic column (50 × 4.6 mm ID) with chemiluminescence detection was developed by Costin et al. [25] for determination of opiate alkaloids in industrial process samples. Monolithic columns exhibit significantly lower backpressure than conventional packed columns and allow much higher flow rates to be applied without a significant decrease in separation efficiency. Therefore, determination of the four predominant alkaloids — morphine, codeine, oripavine, and thebaine — could be achieved in less than 2 min at a flow rate of 3 mL min^{-1}. Further, highly sensitive detection was possible, because of the reduced band broadening and high flow rate, which is closer to the optimum conditions for light production within the detector. Codeine and thebaine were determined using a solvent gradient of ACN in an aqueous solution of trifluoroacetic acid (TFA) and tris(2,2'-bipyridyl)ruthenium (III) chemiluminescence detection. Morphine and oripavine were determined using a solvent gradient of MeOH in an aqueous solution of TFA, and acidic potassium permanganate chemiluminescence. LODs were 1×10^{-10} M, 5×10^{-10} M, 5×10^{-10} M, and 1×10^{-9} M for morphine, codeine, oripavine, and thebaine respectively.

When determination of a broader range of *P. somniferum* alkaloids is required, the high selectivity of chemiluminescence reagents toward distinct groups of alkaloids can be a problem. Therefore, an ion-pair HPLC method with chemiluminescence detection was developed by Lenehan et al. [26] for simultaneous determination of morphine, codeine, oripavine, and thebaine. A dual-function chemiluminescence reagent was prepared by on-line mixing of solutions of tris(2,2'-bipyridyl)ruthenium (II) and acidic potassium permanganate before merging with the column eluent immediately prior to the flow through detector. An additional function of permanganate solution in this system was to oxidize the other reagent to the ruthenium (III) state. Though the sensitivity of the method was lower using the two chemiluminescence reagents in combination than using them individually, it was still superior to UV-absorbance detection for these compounds in industrial process samples.

In the method proposed by Brown et al. [27] to broaden the applicability of chemiluminescence detection further, the column eluent was merged with a formaldehyde solution and then a soluble manganese (IV) reagent. This reagent was less selective than either acidic permanganate or tris(2,2'-bipyridyl)ruthenium (III) and therefore provided a more universal chemiluminescence detection system for HPLC. Six opiate alkaloids were separated within 4 min using monolithic-column HPLC. Not only codeine and thebaine (responsive with tris(2,2'-bipyridyl)ruthenium (III)) and morphine, oripavine, and pseudomorphine (responsible with acidic potassium permanganate) produced a reasonable response with soluble manganese (IV), but in this case papaverine also gave a comparable signal. The LOD for all six alkaloids was approximately 5×10^{-7} mol L^{-1} when an injection volume of 2 µL was used.

30.1.3 HPLC with MS Detection

Though HPLC with UV/diode array detection (DAD) is a powerful and widely used technique to analyze plant extracts, it could provide only very limited structural information like UV spectra; standard compounds are required for identification of individual constituents. The other limiting factor is the poor LOD. In contrast, mass spectra of many compounds are sufficiently specific to allow their identification with a high degree of confidence, if not with complete certainty, and with high sensitivity. The successful application of interfaces to evaporate the HPLC mobile phase and a mixture of polar organic solvents (ACN, MeOH, etc.) in the presence of volatile salts of organic acids (formic acid, acetic acid, etc.) made direct coupling to mass selective detectors possible. The combination of HPLC with MS provides a new tool for the phytochemical analysis of plant extracts, allowing more definitive identification and the quantitative determination of compounds with increased sensitivity.

The fragmentation pathways of the $[M+H]^+$ ions of morphinans were elucidated by Raith et al. [28] after investigations with an ion trap mass spectrometer and a triple-quadrupole system. The electrospray collision-induced dissociation (CID) mass spectra of morphine and codeine display a series of key ions reflecting the substructures of the morphinan skeleton. However, because of the complexity of the CID spectra, an assignment of the different carbon atoms in the morphinan skeleton to these key ions is difficult. Therefore, poppy seeds were allowed to germinate and subsequently to grow in different solutions containing an isotopically labeled precursor, [ring-$^{13}C_6$]-L-tyrosine, [ring-$^{13}C_6$]-tyramine, or [1,2-$^{13}C_2$], [6-*O*-methyl ^{13}C]-(*R,S*)-coclaurine separately. Codeine derivatives ^{13}C labeled to a different extent were isolated and purified from *P. somniferum* seedlings and analyzed by HPLC–electrospray ionization–tandem mass spectrometry (ESI-MS/MS). The CID mass spectra together with the mass shift of these specifically labeled codeine derivatives allowed an unambiguous assignment of the carbon atoms in the morphinan skeleton to the corresponding key ions of these important morphinan alkaloids. Further, Fourier transform ion cyclotron resonance MS (FT-ICR-MS) combined with the infrared multiphoton dissociation (IRMPD) system was used to obtain high-resolution mass data for establishing the elemental composition of important fragment ions of the alkaloid standards morphine and codeine. As a result, all major fragment ions of codeine and morphine were elucidated [29].

High-mass-resolution MS and HPLC-MS/MS were used by Schmidt et al. [30] for elucidation of the alkaloid pattern in *P. somniferum* seedlings after they were fed for a period of nine days with [ring-$^{13}C_6$]-tyramine as a biogenetic precursor of isoquinoline alkaloids. A fast determination of the elemental composition of detected alkaloids, as well as the differentiation of isobaric ions, was possible because of the high-resolving power and mass accuracy of FT-ICR-MS analysis. The incorporation and course of the labeled precursor could be followed very effectively. HPLC-MS/MS analyses were performed using a SepServ RP18 column (5 µm, 100 × 1 mm ID) and gradient elution with ACN/H_2O containing 0.2% acetic acid. The fragmentation behavior of labeled and unlabeled compounds was investigated, yielding a detailed insight into the nature of key ions. About 20 alkaloids belonging to different classes, such as morphinan, benzylizoquinoline, protoberberine, benzo[*c*]phenanthridine, phthalide isoquinoline, and protopine, could be elucidated by comparison with reference compounds in most cases. The combination of these two spectrometric methods was proposed as a powerful tool for investigating *Papaver* alkaloid metabolism.

HPLC and HPLC-MS were applied by Frick et al. [31] for comparative qualitative and quantitative determination of morphinan, tetrahydrobenzylisoquinoline, benzo[*c*]phenanthridine, and phthalideisoquinoline alkaloids in the narcotic cultivar Tasmanian *P. somniferum* and the low-morphine cultivar "Marianne." Latex, leaf, and stem samples were analyzed by HPLC on a LiChrospher 60 RP-select B column (5 µm; 250 × 4 mm ID) with UV detection (at 210, 282, and 440 nm) and dihydrocodeine as an internal standard. The alkaloids were separated at a flow rate of 1 mL min^{-1} with gradient elution using ACN–water containing 0.01% (v/v) phosphoric acid as a mobile phase. Peaks were identified from their UV spectra and by comparison of their retention times with those of authentic standards. The identity of the peaks was confirmed from high-resolution MS data provided by HPLC-ESI-TOF (time-of-flight)-MS after separation on a Superspher 60 RP-select B column (5 µm; 125 × 2 mm ID) using an ACN gradient, with 0.2% (v/v) formic acid as a mobile-phase additive, at a flow rate of 0.2 µL min^{-1}. The alkaloids salutaridine, 1,2-dehydroreticuline, and scoulerine in latex, leaf, and stem samples, as well as noscapine, sanguinarine, dihydrosanguinarine, and their 10-hydroxy-derivatives in the roots, were determined by HPLC-ESI-MS/MS using selected reaction monitoring (SRM) and the reactions leading to the corresponding base peak ion. Morphine, codeine, and thebaine in the latex, and sanguinarine and dihydrosanguinarine in the root system, of the Tasmanian cultivar were present as major alkaloids. A shifted pattern of alkaloid accumulation and reduced levels of total alkaloids were found in the "Marienne" cultivar. Data suggest a differential alkaloid regulation in each cultivar.

30.1.4 Conclusion

HPLC methods for analysis of opium alkaloids have developed tremendously in the past twenty years. HPLC separation and determination of morphinan, tetrahydrobenzylisoquinoline, and phthalideisoquinoline alkaloids from *P. somniferum* on a C_{18} column (5 µm, 120 × 4 mm ID) can be achieved within 15 min using gradient elution and a quaternary mobile phase [18]. Analysis time can be shortened to less than 2 min using a nonporous (micropellicular) stationary phase [16] or monolithic column [25]. Monolithic column HPLC with chemiluminescence detection is becoming more and more important as a rapid, highly sensitive, and relatively inexpensive analytical tool for determination of selected groups of opium alkaloids. HPLC-UV was successfully combined with high-mass-resolution MS and HPLC-MS/MS for comparative qualitative and quantitative determination of alkaloids in transgenic narcotic and condiment *P. somniferum* cultivars, and the obtained data suggested a differential alkaloid regulation in each cultivar. HPLC with MS detection is especially useful for identification of *P. somniferum* alkaloids. Fragmentation pathways and all the major fragment ions of morphine and codeine have been elucidated. Poppy alkaloid profiling was also performed; about 20 alkaloids belonging to different classes could be elucidated. These results were achieved by combined application of ^{13}C-labeled isotopic precursors to seedlings, direct-infusion FT-ICR-MS, and HPLC-MS/MS. These methods together represent a powerful tool that can be used

not only for the determination of fragmentation pathways and ionic structures but also for investigation of *P. somniferum* alkaloid metabolism to obtain information on biosynthetic pathways competing for common precursors and on key enzymatic reactions. This type of information on carbon flow through alkaloid biosynthesis is critical to the design of predictive metabolic engineering.

30.2 PROTOBERBERINE ALKALOIDS AND DERIVATIVES

The HPLC analysis of protoberberine alkaloids and their derivatives including benzo[*c*]phenanthridines, protopines, spiro-benzyltetrahydroisoquinolines, and some related compounds are reviewed in this subchapter (Figure 30.2). Protoberberines are fairly widespread quaternary and tertiary

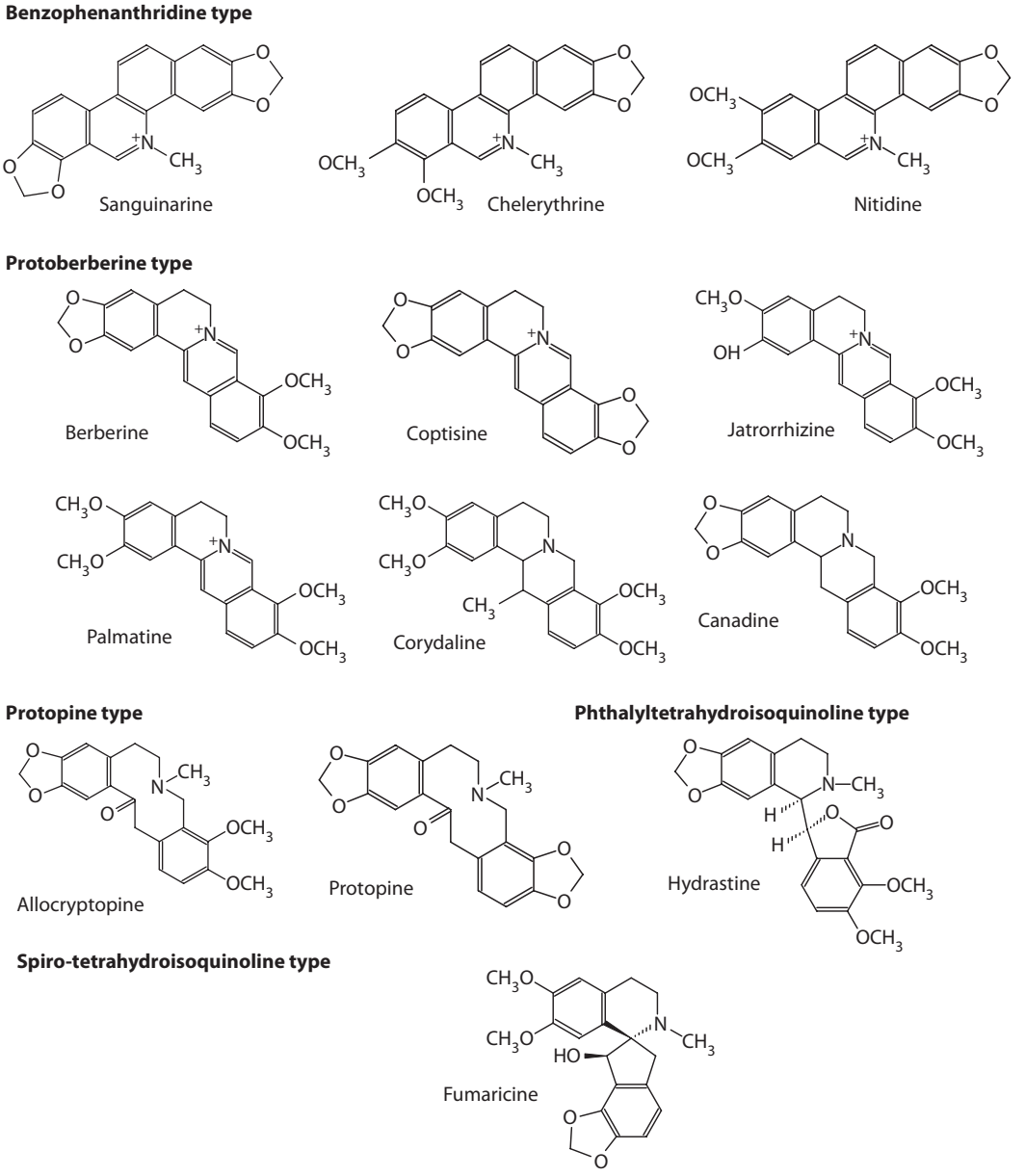

FIGURE 30.2 Selected protoberberine alkaloids and derivatives.

tetracylic alkaloids found in the Berberidaceae, Papaveraceae, Menispermeaceae, Ranunculaceae, and Annonaceae. The isoquinoline skeleton is the basic building block of this type of alkaloids, which are biogenetically derived from tyrosine.

Among the many derivatives arising from the oxidation and rearrangement of tetrahydroprotoberberines, quaternary protoberberines and quaternary benzo[*c*]phenanthridines require a special attention, because they exhibit considerable biological activities [32,33]. Both types of alkaloids display wide antimicrobial, antifungal, and anti-inflammatory activity. They inhibit many enzymes. They also inhibit microtubule assembly and interact with DNA [34–37]. They possess cytotoxic and apoptosis-inducing activity [35,38,39]. The biological effects of these alkaloids are under investigation in many laboratories worldwide.

High performance liquid chromatography (HPLC) combined with DAD, fluorescence detection, or mass spectrometric (MS) detection is the most effective method for separation, identification, and quantification of protoberberine alkaloids due to its enhanced separation efficiency, high sensitivity, and speed. Normal (silica gel) and reversed phases (alkylated silica gels C8, C12, C_{18}, cyanopropyl) [40], and polymeric sorbents (with –CN groups, etc.), can be applied for separation. New-generation columns manufactured from high-purity silica gel with a densely bonded stationary phase can be used to improve peak shape and selectivity [41] and simplify mobile-phase composition [42].

From a chromatographic point of view, the unusual basicity of quaternary benzo[*c*]phenatnthridine alkaloids can be a problem. In alkaline environments, like other typical alkaloids, the cationic forms of sanguinarine (SA) and chelerythrine (CHE) are converted to their free bases. In contrast to the majority of classical alkaloids, however, the bases posses a nonclassical structure with an OH group covalently bonded to carbon C-6 (pseudobases, 6-hydroxy-5,6 dihydroderivatives). pK_R + constants that characterize the acidobasic equilibrium between the cationic and neutral (pseudobase) forms of SA and CHE are between 7 and 9 [43]. Therefore, when analysis is performed at a moderately acidic or neutral pH, both charged and uncharged forms of these alkaloids exist, which can be a source of irregular peak shape. Further, free bases (and not the charged forms) of SA and CHE are capable of nonbonding interaction with compounds bearing negatively charged groups, simple mercapto compounds, SH enzymes, and human serum albumins at physiological pH (pH 7.4), as has been shown. Further, these pseudobases tend to form dimers through an ether bond in a weakly alkaline medium. These capabilities of free bases can be among the reasons for SA and CHE's strong interactions with the stationary phase that can be observed at a moderately acidic or neutral pH (Figure 30.3) [42,44]. The pseudobases of the quaternary protoberberine alkaloids

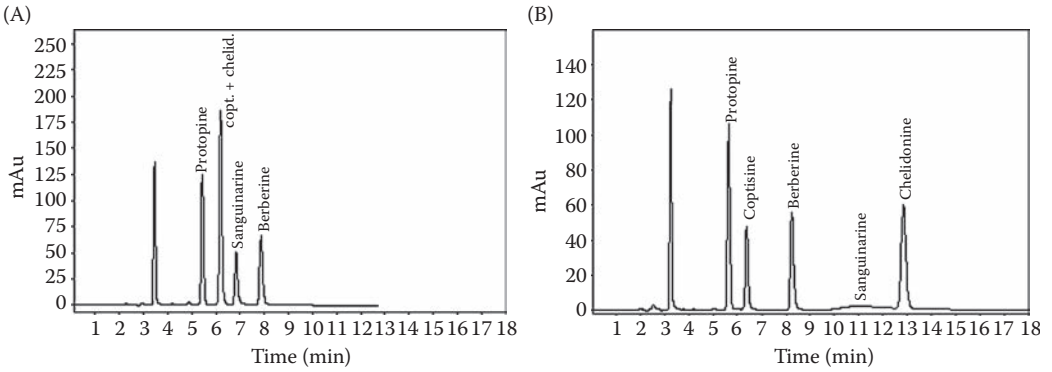

FIGURE 30.3 HPLC separation of alkaloid standards at (A) pH 3.80 and (B) pH 5.60. Luna C_{18}(2) column. Mobile phase 45:60 (v/v) acetonitrile–50 mM ammonium formate containing 0.25% decanesulfonic acid sodium salt. Flow rate 1 mL min^{-1}. Detection at 280 nm. (Reprinted from Kursinszki, L., Sárközi, Á., Kéry, Á., and Szőke, É., *Chromatographia* 63, S131–S135, 2006. With permission from Friedr. Vieweg & Sohn/GWV Fachverlage GmbH.)

beberine and coptisine, unstable 8-hydroxy adducts, cannot cause such a problem, because they exist only in a strongly alkaline medium (pH > 12).

30.2.1 Sample Preparation

Sample preparation is the crucial first part in a phytochemical analysis method because it is necessary to extract the desired chemical components from the herbal material, dissolve the analyte in a suitable solvent, and remove as many interfering compounds as possible from the solution. Particular attention has to be given to the critical steps of sample preparation, because they may be possible sources of imprecision, especially at very low concentrations of substances of interest. Several sample preparation protocols have been described for the HPLC analysis of protoberberine alkaloids and derivatives. These are summarized in Tables 30.2 through 30.4, and only the most important methods will be reviewed here.

Four extraction methods (maceration, microwave-assisted extraction [MAE], ultrasound-assisted extraction [UAE], and percolation) for SA and CHE in the dried fruits of *Macleaya cordata* were investigated and compared by Zhang et al. [45] (Table 30.2). MAE proved to be the most effective method, capable of yielding SA and CHE with a short extraction time. Both pressurized liquid extraction and multiple ultrasound-assisted extractions provide significant time savings over Soxhlet extraction of hydrastine and berberine from *Hydrastis canadensis* and yield comparable results [46]. Dried and powdered plant samples could also be extracted with acetic acid in ultrasound-assisted extraction (Table 30.2) [19].

An accelerated solvent extraction (ASE) method was developed by Chen et al. [47] for major alkaloids (seven protoberberines and magnifolin) in the dried root of *Coptis chinensis*. The operational parameters including solvent composition, extraction temperature, static extraction time, and extraction cycles were optimized. Further, ASE was compared with reflux extraction and ultrasonic extraction, and the shorter extraction time was found to be the main advantage of ASE over the other two methods.

Quaternary alkaloid–containing fractions may be efficiently concentrated by means of ion-pair extraction using sodium perchlorate [48]. Eight quaternary alkaloids in *Zanthoxylum usambarense* were thus obtained, one of which was a new quaternary base of the tetrahydroprotoberberine type, which was named (–)-usambarine [49].

Ion-pair solid-phase extraction (SPE) using *n*-heptanesulfonic acid (HEPES) was applied for isolation of alkaloids from acified methanolic extracts of *Sanguinaria canadensis* cell cultures [50] and commercial tinctures of *Chelidonium majus* [42]. Samples were diluted four times with HEPES (0.05 M) aqueous solution and centrifuged, and the supernatant was applied on an SPE C_{18} microcolumn. After column washing with methanolic HEPES solution, the alkaloids were eluted with HEPES in 95% methanol (Table 30.2). SPE method recoveries in the range of 98.8–102.4% were obtained for protopine, chelidonine, sanguinarine, and berberine in a methanolic standard solution [42].

Preconcentration and isolation of major benzophenanthridine alkaloids from cell culture medium of *Eschscholtzia californica* was achieved by SPE on a Phenomenex Strada SPE C_{18}-E cartridge preconditioned with 0.5% acidified methanol and then water. After sample application and column washing with 10% acidified methanol in water, alkaloids were eluted with 0.5% acidified methanol (Table 30.2) [51].

Bioassay guided fractionation by positive-pressure column chromatography over silica gel resulted in the isolation of SA and CHE from crude methanolic extracts of *Sanguinaria canadensis* roots [52]. A column chromatographic pseudo-reversed-phase system, unmodified silica gel with methanol in an acetate buffer of pH 6.0, was applied by Golkiewicz and Gadzikowska [53] for separation of tertiary and quaternary alkaloids (SA, CHE, dihydro derivatives of SA and CHE, and chelilutine, chelirubine, and berberine) from *Chelidonium majus* into two groups. The retention of alkaloids in such a system can be controlled by changing the concentration of methanol or the pH of the mobile phase. Quaternary alkaloids were separated further into fractions containing two or

TABLE 30.2
Reversed-Phase HPLC Methods for Analysis of Protoberberine Alkaloids and Derivatives

Alkaloids	Adlumidiceine Coptisine Cryptopine Parfumine Protopine	Chelirubine Sanguinarine Chelerythrine	Chelirubine Sanguilutine Sanguinarine Sanguirubine	Chelerythrine Norsanguinarine Chelilutine	Tertiary and quaternary alkaloids
Aims	*Fumaria parviflora* *Fumaria capreolata*	*Sanguinaria canadensis* Cell culture	*Sanguinaria canadensis* *Eschscholtzia californica* *Papaver somniferum* Cell cultures		*Chelidonium majus*
Reference	**58. Válka and Šimánek 1988**	**50. Chauret et al. 1990**	**65. Chauret et al. 1991**		**44. Han et al. 1991**
Sample preparation **Extraction**	Soxhlet extractor — air-dried whole plants — methanol. Concentrated in vacuo, acidified with acetic acid and washed with diethyl ether solid sodium carbonate pH 8 extraction with diethyl ether fraction A sodium hydroxide pH 12 extraction with diethyl ether fraction B acidified with hydrochloric acid pH 5 + solid potassium iodide and extraction with chloroform fraction C	Cells were disrupted (room temperature) into 20 mL acidic methanol (0.5% HCl, v/v), centrifuge at 12000 g 15 min, re-extract 20 mL acidic methanol. Biomass crude extract and culture-medium: dilute with 0.05 M *n*-heptanesulfonic acid aqueous solution (HS). **SPE:** reversed-phase C_{18} cartridge (400mg Sep-Pak) Adsorbent preparation: 5 mL acetonitrile, 5 mL 5% HS (0.05 M) in methanol, and 5 mL 100% HS. Wash with 10 mL 25% HS (0.05 M) solution in methanol, air dry. Recover the alkaloids in 2 mL 5% HS (0.05 M) in methanol. **LLE:** Culture medium: pH 9–10 with sodium hydroxide, extract with ethyl acetate (3 × 30 mL), evaporate to dryness (35°C nitrogen), dissolve in 2 mL acidic methanol. Biomass crude extracts: evaporate to dryness, dissolve in 25 mL 1 M HCl, and wash two times with 25 mL ethyl acetate followed by 25 mL diethyl ether. Filter 0.45 µm	Cells were disrupted (room temperature) into 20 mL acidic methanol (0.5% HCl, v/v), centrifuge at 12000 g 15 min, re-extract in 20 mL acidic methanol. Filter 0.45 µm Acidify 10 µL sample with 2 µL acidic methanol (33%, v/v, phosphoric acid)		Plants were dried at room temperature — extracted in ethanol. Distill off ethanol. Dissolve residue in 1% sulphuric acid — filter. Neutralization with sodium hydroxide and extraction with chloroform. pH increase to 8 — extraction with chloroform, pH increase by 1 unit and extract — repeat until pH 14. Combine extracts, filter, and evaporate to dryness under vacuum. Dissolve in 1% hydrochloric acid filter and evaporate to dryness under vacuum. Dissolve in methanol + 0.1 M ammonia
Column **L x ID (mm)**	Separon SGX C_{18} 7 µm 250 × 4	Guard column: 15 × 3.2, 7 µm Spheri-5 cyano 5 µm 100 × 4.6; two columns in series Temperature: 40°C	Guard column: 15 × 3.2, 7 µm Spheri-5 cyano 5 µm 100 × 4.6; two columns in series Temperature: 40°C		Hypersil ODS C_{18} 5 µm 100 × 4.6 Temperature: 30°C

HPLC mobile phases	A: methanol–water–triethylamine (50:50:0.1 to 80:20:0.1, v/v/v, in 10 min) B: 50 mM triethylammonium phosphate buffer (pH 4.0) in methanol–water (52:48, v/v)	Methanol–water (86:14, v/v) + triethylamine (5 mM) + phosphoric acid (5 mM) pH 5.6 with sodium hydroxide	Methanol–water (86:14, v/v) + triethylamine (5 mM) + phosphoric acid (5 mM) pH 5.5 with sodium hydroxide	Water–acetonitrile–methanol (50:20:30 to 15:55:30 in 15 min) pH 7.5 with propylamine methanol contained 0.15 M potassium iodide
Flow rate (mL min^{-1})	1	1	1	Increased from 0.8 to 1.5 in 15 min
Detection (λ) (nm)	357 for coptisine 280 for the other alkaloids	Fluoresce: 338 UV: 280	Fluoresce: 225 excitation 410 emission UV: 280	285 (bandwidth: 30) Reference wavelength: 400 (bandwidth: 100)
Limit of detection (ng)	—	Sanguinarine: 0.8 Chelirubine: 0.4 Chelerythrine: 5.0 (fluoresce), 1.6 (UV)	Chelirubine: 0.1 Sanguilutine: 0.4 Sanguinarine: 0.2 Sanguirubine: 0.2 Chelerythrine: 0.7 Norsanguinarine: 0.4 Chelilutine: 0.5	—
Alkaloids	Sanguinarine Chelerythrine	Opium alkaloids Morphine Papaverine Codeine Noscapine Oripavine Sanguinarine Thebaine	Isoquinoline alkaloids Protopine Sanguinarine Chelidonine Berberine Coptisine	Sanguinarine Chelirubine Macarpine Chelerythrine Chelilutine
Aims	*Macleaya cordata* Fruit	*Papaver somniferum* *P. setigerum* *P. bracteatum* *P. pseudo-orientale*	*Chelidonium majus*	*Eschscholtzia californica* Cell cultures
Reference	**45. Zhang et al. 2005**	**19. Yoshimatsu et al. 2005**	**42. Kursinszki et al. 2006**	**51. Klvana et al. 2006**
Sample preparation Extraction	Maceration: aqueous hydrochloric acid solution (0.1 mol L^{-1}), water bath 30 min. Ultrasound-assisted: aqueous hydrochloric acid solution (0.1 mol L^{-1}), 30 min, power: 250W ultrasonic bath. Microwave-assisted: aqueous hydrochloric acid solution (0.1 mol L^{-1}), 5 min, power: 700W Percolation: glass column (700 × 25 mm), 500 mL aqueous hydrochloric acid solution (0.1 mol L^{-1}), 2mL L^{-1}, 25°C Filter 0.45 μm membrane	Plant materials, air-dried or lyophilized, powdered, were extracted with different solvents, under sonication for 30 min. Vortex 1 min, centrifuged (18000 × g) 10 min. **LLE:** powdered capsules + 5% acetic acid (under sonication), filtrate, wash with acid chloroform, alkaline with 28% ammonia, and extract with chloroform–isopropanol (3:1) 3 times, concentrate to dryness, and dissolve in 50% aqueous methanol. **SPE:** RP-SPE cartridge + cation-exchange RP-SPE cartridge. extraction solvent: water, 5% acetic acid, 0.1 M sodium citrate buffer (pH 6.0) conditioned with methanol, equilibrated with water, concentrated to dryness, and dissolved in 50% aqueous methanol	Plants were dried, powdered, and extracted with methanol containing 0.05 M hydrochloric acid by sonication (27°C, 2 × 10 min). Centrifugation and evaporated to dryness. **SPE**: Supelclean LC-18 column. Samples were dissolved in 1.25 mL methanol containing 0.05 M hydrochloric acid, diluted with 3.75 mL 0.05 M *n*-heptanesulfonic acid aqueous solution (HS). Activation: 5 mL acetonitrile, 5 mL 5% HS (0.05 M) in methanol, 5 mL 100% HS. Wash: 5 mL 70% HS (0.05 M) in methanol. Elute: 2.5 mL 5% HS (0.05 M) in methanol.	Cell suspensions (10 mL) filtered and washed twice with 20 mL water under vacuum. Extract overnight in 0.5% acidified methanol, then sonicate for 30 min. Centrifuge. Evaporate to dryness 4 mL supernatant, resuspend in 400 μL 0.5% acidified methanol. Filter through 0.45 μm membrane. **SPE:** Phenomenex Strata C_{18}-E column. Sample: 10 mL cell medium filtered Condition: 3 mL 0.5% acidified methanol + 3 mL water Wash: 10% acidified methanol Elute: 1 mL 0.5% acidified methanol Filter 0.45 μm membrane

(*Continued*)

TABLE 30.2 CONTINUED)
Reversed-Phase HPLC Methods for Analysis of Protoberberine Alkaloids and Derivatives

Column **L x ID (mm)**	Johnson spherigel analytical column C_{18} 5 µm 250 × 4.6 Temperature: 30°C	TSK gel ODS-120 A 250 × 4.6 Temperature: 40°C	Phenomenex SequrityGuard C_{18} guard column (8 × 3) Phenomenex Luna C_{18} (2) 5 µm 250 × 4.6		Securiguard C_{18} guard column Zorbax Eclipse XDB-C_{18} 5 µm 250 × 4.6 Temperature: 35°C
HPLC mobile phases	A: 0.05 mol L^{-1} sodium dihydrogen phosphate buffer (pH 3.0 with H_3PO_4) B: acetonitrile Isocratic elution A + B = 65 + 35 (v/v) Gradient elution: 0–20 min, 80% A; 20–25 min, 80%–65% A; 25–30 min, 65% A	A: Acetonitrile B: 10 mM sodium 1-heptanesulfonate in water, pH 3.5 with phosphoric acid Gradient: 0–15 min, 20%–30% A; 15–20 min, 30%–40% A; 20–25 min, 40% A; 25–30 min, 40%–50% A; 30–35 min, 50%–20% A	Acetonitrile–methanol–30 mM ammonium formate, pH 2.80 (14.7:18:67.3, v/v/v) Temperature: 30°C		A: 50 mM phosphoric acid (pH 3.0 with potassium hydroxide) B: acetonitrile 0–2 min, 25% B; 2–12 min, linear gradient to 35% B; 12–14 min, 35% B; 14–22 min, linear gradient to 80% B; 22–29 min, 80% B; 29–31 min, linear gradient to 25% B; 31–33 min, 25% B
Flow rate (mL min^{-1})	1	1	1		1.5
Detection (λ) (nm)	270	284	280		Fluorescence: ex. 330 – em. 570 UV: 250–450 (341)
Limit of detection (ng)	—	—	Protopine: 0.5 Sanguinarine: 0.4 Chelidonine: 0.5	Berberine:0.5 Coptisine: 0.2	—

Note: LLE, liquid–liquid extraction; SPE, solid-phase extraction.

three alkaloids using gradient elution with methanol as an organic solvent. SA and CHE of high purity were obtained then by separation of these fractions using micropreparative zone thin-layer chromatography (TLC) [54].

30.2.2 Reversed-Phase HPLC

Reversed-phase HPLC (RP-HPLC) applying alkylated silica gel columns (C8, C12, C_{18}) is currently the most popular method for analysis, because of its high-separation power allowing simultaneous determination of a variety of protoberberine alkaloids and derivatives with different physicochemical properties. Most of these alkaloids contain strong UV-VIS chromophores; therefore, highly sensitive UV-VIS detection or DAD can be applied, or, alternatively, more selective and sensitive fluorometric detection can be used.

An RP-HPLC method on a Nucleosil C_{18} column (150 × 4.6 mm ID) using a linear gradient with water–ACN–H_3PO_4 was applied by Tanahashi and Zenk [55] for the fingerprint analysis of benzo[*c*]phenanthridine alkaloids (dihydro- and hydroxydihydro- derivatives of SA, CHE, and chelirubine; dihydromacarpin and dihydrochelilutin) from the elicited cell cultures of *Eschscholtzia californica*. Production of SA by *Chelidonium majus* callus cultures was investigated by Colombo and Tome [56]. Alkaloids were extracted in a Soxhlet apparatus with 70% ethanol, and the reduced alcoholic extract was purified by ion-pair liquid–liquid extraction. Chromatographic separation was achieved on a Lichrospher C_{18} column (5 µm; 250 × 4 mm ID) using gradient elution with ACN–MeOH + 0.001% H_3PO_4. SA concentration was measured at 280 nm, and the highest value was 15 µg g^{-1} dry weight.

Twenty peaks were resolved, and 13 tertiary and quaternary alkaloids were identified in extracts of *Chelidonium majus* by RP-HPLC on an ODS Hypersil column with gradient elution using water–ACN–MeOH (from 5:2:3 to 3:11:6 in 15 min). The water was adjusted to pH 7.5 with propylamine, and the methanol contained 0.15 mM potassium iodide. KI added to the mobile phase improved resolution considerably, whereas previously investigated organic amine modifiers gave unsatisfactory results (Table 30.2) [44]. Different salts (Na^+, NH_4^+, K^+, combined with phosphate, acetate, Br^-, SCN^-, I^-) added to the mobile phase eliminated peak tailing and decreased retention of cationic forms of chelidonium alkaloids due to a silanol-masking effect. The extended donor-acceptor concept was used to explain the effectiveness of different salts in blocking the active sites of the stationary phase [57].

Válka and Šimánek [58] reported the determination of alkaloids of *Fumaria parviflora* and *F. capreolata* by HPLC and capillary isotachophoresis (ITP). HPLC separation of tertiary alkaloids (adlumidiceine, cryptopine, parfumine, protopine) was achieved on a Separon SGX C_{18} (7 µm) column (250 × 4 mm ID) with the mobile phase MeOH–water–TEA, from 50:50:0.1 (v/v/v) to 80:20:0.1 in 10 min, but the quaternary alkaloid coptisine showed tailing under those conditions. Therefore, 50 mM triethylammonium phosphate buffer (pH 4.0) in MeOH–water (52:48, v/v) was used as a mobile phase for its determination (Table 30.2). HPLC and ITP yielded comparable results for alkaloid contents and precision.

Soušek et al. [59] determined 60 tertiary and quaternary alkaloids (protoberberines, spirobenzyltetrahydroisoquinolines, and some others) in *Fumaria officinalis* and seven other *Fumaria* species using RP-HPLC on a Hypersil ODS C_{18} column (5 µm; 250 × 4.6 mm ID) with ACN–triethylammonium phosphate gradient elution. The peaks of all the alkaloids were resolved within 25 min, and only sinactine and protopine were not baseline separated. As expected, the type of substituents had an influence on the t_R of the alkaloids: The retention time decreased as a function of hydroxyl group substitution. Further, quaternary alkaloids eluted later than tertiary bases, which was probably due to their higher hydrophobicity. In the *Fumaria* species investigated, adlumidiceine, fumaricine, fumariline, *O*-methylfumarophycine, fumarophycine, *N*-methylstylopine, parfumine, and protopine were found to be the major alkaloid components.

An internally standardized RP-HPLC method was developed by Chu and Sheu [60] for the analysis of alkaloids in the coptis-evodia herb couple (*Coptidis rhizome* and *Evodiae fructus*, 1:3, g/g).

When a mixture of MeOH and sodium acetate–acetic acid buffer alone or supplemented with sodium dodecyl sulfate (SDS) was used as a mobile phase, the former offered sharper peak shape, higher resolution, and a more stable retention time; therefore, it was chosen for separation of alkaloids. Seven quaternary and 10 tertiary alkaloids were separated on a Cosmil 5 C_{18}-MS column (5 μm; 250 × 4.6 mm ID) and determined at 250 nm with LODs of 0.28–6.77 ng. The HPLC method was proposed for the quality control of Chinese herbal preparations containing the coptis-evodia herb couple.

An externally standardized RP-HPLC method has been described by Abourashed and Khan [61] for determination of berberine and hydrastine in dietary supplements containing goldenseal (*Hydrastis canadensis*). Separations were achieved within 20 min on a Phenomenex Luna C_{18} column with gradient elution using ACN, MeOH, and water containing 100 mM sodium acetate and acetic acid (pH 4.0) as solvents. Method recovery was 98.38% for (–)-β- hydrastine and 98.36% for berberine. The LOQ was 3.13 μg mL^{-1}, and the LOD was 1.00 μg mL^{-1} for each alkaloid. The analyzed samples exhibited a wide range of alkaloid concentrations, from 0 to 2.51% for hydrastine and from 0 to 4.35% for berberine.

An RP-HPLC method with a mobile phase free from ion-pair reagents and triethanolamine has been described by Kursinszki et al. [42] for simultaneous determination of isoquinoline alkaloids (SA, protopine, chelidonine, coptisine, and berberine) in the aerial parts of *Chelidonium majus* (Table 30.2). Crude extracts were prepared with acidic methanol and purified by SPE on Supelclean LC-18 cartridges. Alkaloids were separated on a C_{18} reversed-phase column. Excellent peak shapes were obtained using Luna C_{18}(2), a new-generation silica-based stationary phase with ACN–MeOH–30 mM ammonium formate, pH 2.80, as a mobile phase (Figure 30.4). The LODs of these alkaloids ranged from 0.6 to 1.5 ng. The method was further used to control results obtained by TLC [62].

Sanguinarine in argemone and other adulterated edible oils was rapidly determined by a simple and reliable RP-HPLC method developed by Husain et al. [63]. Oil samples were diluted 10 times with the mobile phase MeOH–ACN–THF–water (21:55:4:20, v/v) and were separated on a C_{18} column within 11 min. Quantification was done at 280 nm in the range of 0.01–1.0 mg g^{-1} with the detection limit of 5 mg g^{-1}.

RP-HPLC methods were used for achiral and chiral determination of dihydrosanguinarine and its 6-methoxy- and 6-acetonyl- derivatives in methanol extracts of *Hylomecon hylomeconoides* and

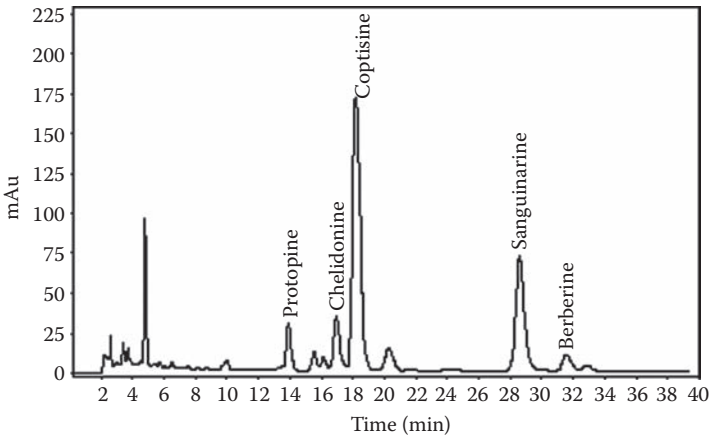

FIGURE 30.4 HPLC chromatogram obtained from an extract of the aerial parts of *Chelidonium majus*. Luna C_{18}(2) column. Mobile phase 14.7:18:67.3 (v/v) acetonitrile–methanol–30 mM ammonium formate, pH 2.80. Flow rate 1 mL min^{-1}. Detection at 280 nm. (Reprinted from Kursinszki L., Sárközi, Á., Kéry, Á., and Szőke, É., *Chromatographia* 63, S131–S135, 2006. With permission from Friedr. Vieweg & Sohn/GWV Fachverlage GmbH.)

H. vernale (Papaveraceae). Achiral RP-HPLC separation and quantification of these alkaloids was achieved on an ODS column using ACN and 50 mM phosphate buffer, pH 7.0, as a mobile phase. The analysis time was 20 min. The same amount of (+) and (–) enantiomers of 6-methoxydihydrosanguinarine was found using chiral columns [64].

Fluorometric detection ($\lambda_{ex} = 225$ nm, $\lambda_{em} = 410$ nm) was applied by Chauret et al. [50] for the identification and determination of benzophenanthridine alkaloids (SA, CHE, chelirubine, chelilutine, sanguilutine, sanguirubine, and norsanguinarine) in extracts from cell cultures of *Sanguinaria canadensis* (Table 30.2). HPLC separation was achieved on two Spheri-5 cyano columns (5 µm; 150 × 4.6 mm ID) in series, with a mobile phase consisting of MeOH and an aqueous solution containing 5 mM TEA and 5 mM H_3PO_4 adjusted to pH 5.6 with sodium hydroxide. The high selectivity of fluorescence of benzophenanthridine alkaloids allowed direct RP-HPLC analysis of crude cell extracts and medium samples without any further purification. LODs ranged from 0.1 to 0.7 ng [65].

An RP-HPLC method (Table 30.2) was developed by Klvana et al. [51] for evaluation of the benzophenanthridine alkaloid production of *Eschscholtzia californica* cell cultures. Sanguinarine, chelirubine, macarpine, chelerythrine, and chelilutine were separated on a Zorbax Eclipse XDB-C_{18} column using gradient elution with ACN and 50 mM H_3PO_4 adjusted to pH 3 with sodium hydroxide. Both fluorescence and photodiode array detection were performed, leading to an improvement in selectivity and enabling simplification of the sample-preparation protocol. Methanolic extraction was suitable for cultured cells, and SPE was required only for medium samples; the extraction yield was above 80% in both cases.

30.2.3 Ion-Pair HPLC

Due to the ionic character of protoberberine alkaloids and their derivatives in an acidic medium and their tendency to interact with alkylated silica gel columns, ion-pair chromatography is frequently applied. Organic amine modifiers (triethyl-, tetrabutylamine) and ion-pair reagents (sodiumdodecylsulfate, alkylsulfonic acids, i.e., heptane-, hexane-, and octanesulfonic acids) are used to avoid the ionic interactions forming stable ion association with positively charged forms of alkaloids. Peak tailing can be reduced, and retention of cationic species can be decreased, due to silanol-masking effects, by addition of salts (Na^+, NH_4^+, K^+, combined with phosphate, acetate, Br^-, SCN^-, I^-) as well.

Ion-pair HPLC with an SDS-supplemented (0.5%) mobile phase composed of 0.05 M tartaric acid–MeOH–ACN (44:10:46, v/v) was applied by Niu and He [66] for determination of eight isoquinoline alkaloids in extracts of the subterranean parts of *Chelidonium majus* (Table 30.3). Alkaloids were separated on an ODS YWG-C_{18} (10 µm) column (250 × 4 mm ID) and measured, with LODs of 10 ng for chelidonine, 24 ng for berberine, 5 ng for protopine, 10 ng for coptisine, and 4–6 ng for tetrahydrocoptisine, 6-methoxydihydrochelerythrine, 6-methoxydihydrosanguinarine, and dihydrosanguinarine. Detection was at 290 nm, and the analysis time was 40 min.

An ion-pair HPLC method was applied by Reinhart et al. [67] for determination of sanguinarine in extracts from *Macleaya* ssp. and SPE-purified samples of saliva and gingival cervical fluids (Table 30.3). Analysis was performed on an ethyl silane column with acidic and basic ion-pair reagents together in the mobile phase because a significant tailing, possibly due to sanguinarine's mixed mode of retention, was observed in the chromatogram when a cation-pairing reagent alone was used (Table 30.3). The LOD was 3 ng for sanguinarine in samples.

An HPLC method using sodium lauryl sulfate and tartaric acid in ACN–water as the eluent has been described for determination of isoquinoline alkaloids in *Argemone ochroleuca* and *A. mexicana* seeds after their extraction into weakly acified methanol. *A. ochroleuca* contained dihydrosanguinarine and dihydrochelerythrine (ca. 3:2) as major and SA, CHE, protopine, and berberine as minor alkaloid components. *A. mexicana* contained only dihydrosanguinarine as a major and SA and berberine as minor alkaloids, in agreement with earlier studies. Dihydrosanguinarine and dihydrochelerythrine were measured as their oxidized products, SA and CHE, after UV irradiation [68].

TABLE 30.3
Ion-Pair HPLC Analysis of Protoberberine Alkaloids and Derivatives in Plants

Alkaloids	Sanguinarine	Isoquinoline alkaloids Tetrahydrocoptisine 6-Methoxydihydrochelerythrine 6-Methoxydihydrosanguinarine Dihydrosanguinarine	Chelidonine Berberine Protopine Coptisine	Isoquinoline alkaloids Main and minor alkaloids	Chelirubine Macarpine Sanguilutine Berberine Sanguirubine Protopine	Sanguinarine Coptisine Chelerythrine Chelilutine Allocryptopine Stylopine Magnoflorine
Aims	*Macleaya microcarpa* and *M. cordata* Saliva, gingival crevicular fluid Determination of sanguinarine	*Chelidonium majus* Separation and determination of alkaloids		*Chelidonium majus* Separation and identification of alkaloids	Papaveraceae Determination of alkaloids in seven plant species	
Reference	**67. Reinhart et al. 1991**	**66. Niu and He 1991**		**70. Táborská et. al. 1994**	**41. Suchomelová et al. 2007**	
Sample preparation	Acidified methanolic extraction	Plants were dried, powdered, and immersed in 5 mL chloroform–ethanol (1:1), then macerated for 12 h and ultrasonicated for 30 min.		Plant materials air-dried at room temperature,	Root extract obtained by maceration (3–4 days) with methanol, diluted 10 times with the mobile phase	
Extraction	*Solid-phase extraction:* conditioned: methanol, then water washed: water elute: 0.5% HCl–methanol (v/v)	After clarification the upper solution was retained and internal standard (2 ng/μL) was added.		extracted with methanol (six times/six days) in Soxhlet Filter extract, concentrate, dilute 10 times with phosphate buffer, pH 3		
Column **L x ID (mm)**	Apex I ethyl column 5 μm 250 × 4.6	ODS silica gel YWG-C_{18} 10 μm 250 × 4.0 Temperature: ambient (20–25°C)		TSK gel ODS-120 Å 250 × 4.6	Phenomenex C12 Synergi™ Max-RP 80A 4 μm 150 × 4.6	

HPLC mobile phases	Water (2.75 mmol/L hexanesulfonic acid – 2.25 mmol/L hexyltriethylammonium phosphate)–acetonitrile (60: 40, v/v) pH 2.7	0.05 M tartaric acid–methanol–acetonitrile (44:10:46, v/v/v) + 0.5% sodium dodecyl sulphate	Diluting solvent: water (0.01 mol L^{-1} heptanesulfonic acid + 0.1 mol L^{-1} triethylamine, pH 2.5 with phosphoric acid) A: 25% acetonitrile (v/v) B: 60% acetonitrile (v/v) Elution profile: 0–2 min, linear gradient 0%–10% B in A; 2–3 min, linear gradient 10%–20% B in A; 3–22 min, isocratic 20% B in A; 22–30 min, linear gradient 20%–100% B in A; 30–60 min, isocratic 100% B	Diluting solvent: Heptanesulfonic acid (0.01 M) + triethylamine (0.1 M) in water, pH 2.5 (H_3PO_4) A: 25% acetonitrile (v/v) B: 60% acetonitrile (v/v) Elution profile: 0–1 min, 20% B in A; 1–10 min, 50% B in A; 10–20 min, 100% B in A; 20–25 min, isocratic 100% B
Flow rate (mL/min)	3	1	0.5	0.5
Detection (λ) (nm)	280 328	290	280	280
Limit of detection (ng)	Sanguinarine: 3	—	—	Chelirubine: 64.00 Macarpine 33.00 Sanguilutine: 9.40 Berberine: 17.00 Sanguirubine: 68.00 Protopine: 6.00 Sanguinarine: 1.70 Coptisine: 34.00 Chelerythrine: 23.00 Chelilutine: 10.00 Allocryptopine: 20.20 Stylopine: 12.00 Magnoflorine: 9.00

A comparative analysis of the alkaloid composition in populations of *Eschscholtzia californica* of different origins was carried out by Tomè et al. [69] using the aerial parts of plants grown in a phytothron. RP-HPLC separations were achieved on a Lichrosper C8 column (5 μm; 250 × 4 mm ID) using gradient elution with water–ACN containing 10 mM octylsulfonic acid (sodium salt) and 0.15 M TEA adjusted to pH 3 with H_3PO_4. Seven alkaloids (*O*-methylcaryachine, protopine, α-allocryptopine, eschscholtzine, californidine, SA, and CHE) were detected and determined using external standards in the range of 0.01–0.5 mg mL^{-1}. The major components were the pavine alkaloids eschscholtzine and californidine in all samples.

A mixture of 28 alkaloid standards was used by Táborská et al. [70] as a model of the *Chelidonium majus* extract composition (Table 30.3). Satisfactory RP-HPLC separation of 27 alkaloids of this mixture was achieved within 52 min with a complex gradient program using acetonitrile and an aqueous solution of 10 mM heptanesulfonic acid (10 mM) and TEA (100 mM), adjusted to pH 2.5 with H_3PO_4. Twenty-one compounds were separated and identified from both the aerial and underground parts of *C. majus*, which contained coptisine as the main constituent. However, dihydrochelirubin and magnoflorin were found only in the roots, and chelamine and chelamidine were detected only in the aerial part.

Seven quaternary benzophenanthridine alkaloids were quantified by Suchomelová et al. [41] in the underground part of six plant species of the family Papaveraceae (*Sanguinaria canadensis*, *Dicranostigma lactucoides*, *Chelidonium majus*, *Macleaya cordata*, *M. microcarpa*, and *Stylophorum lasiocarpum*). Sanguinarine, chelerythrine, chelirubine, chelilutine, sanguilutine, sanguirubine, and macarpine were analyzed by HPLC on a Phenomenex Synergy™ Max-RP C-12 column (5 μm; 150 × 4.60 mm ID) with a mobile phase consisting of heptanesulfonic acid (0.01 M) and TEA (0.1 M) in water, pH 2.5, and ACN in a gradient from 25% to 60% during 25 min (Figure 30.5, Table 30.3). Improved separation and sharp peaks were obtained for isoquinoline alkaloids, which was due to the use of the Synergy Max-RP bonded phase, which provided better surface coverage and reduced silanol activity. Alkaloids were measured at 280 nm, and the LOD was 1.7 ng mL^{-1} for sanguinarine and 23 ng mL^{-1} for chelerythrine at an injection volume of 50 μL. Among the plant species investigated, *Dicranostigma lactucoside* contained the highest amount of sanguinarine (1.99%) and chelerythrine (3.43%).

30.2.4 HPLC with MS Detection

HPLC and liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC-APCI-MS) were applied by Iwasa et al. [71,72] for qualitative analysis of metabolites of protoberberine alkaloids in extracts obtained from feeding experiments in cell cultures of *Corydalis pallida* and the biogenetic pathway from the protoberberines was defined. Alkaloids were separated on Cosmosil 5 C_{18}-AR (150 × 4.6 mm ID) using gradient elution with 0.1 M ammonium acetate (0.05% TFA) and MeOH. Tetrahydroprotoberberines and protopines showed quasimolecular ions $[M + H]^+$, quaternary alkaloids such as the protoberberberinium salts cluster ions $[M + CF_3]^+$, and quaternary alkaloids such as the benzophenanthridines and the α- and β-*N*-metho salts of tetrahydroprotoberberines molecular ions $[M]^+$, under the LC-APCI-MS conditions applied.

Ion-pair HPLC-ESI-MS/MS was used by Fabre et al. [73] for direct characterization of isoquinoline alkaloids in a crudely purified extract of *Eschscholtzia californica* after separation on a Hypersil C8 column (5μm; 150 × 4.6 mm ID) using gradient elution with ACN and water containing 1 mM SDS and 10 mM TEA adjusted to pH 2.5 with H_3PO_4 (Table 30.4). Fourteen isoquinoline alkaloids, including pavines, benzylisoquinolines, aporphynes, protopines, and benzophenanthridines, were detected and characterized. In MS/MS experiments, characteristic diagnostic ions were observed together with similar neutral and/or radical losses for these different compounds, and both the similarity and diversity of the structures detected allowed an explanation for these degradations to be suggested by crossing the data and thus to enhance the specificity of the method.

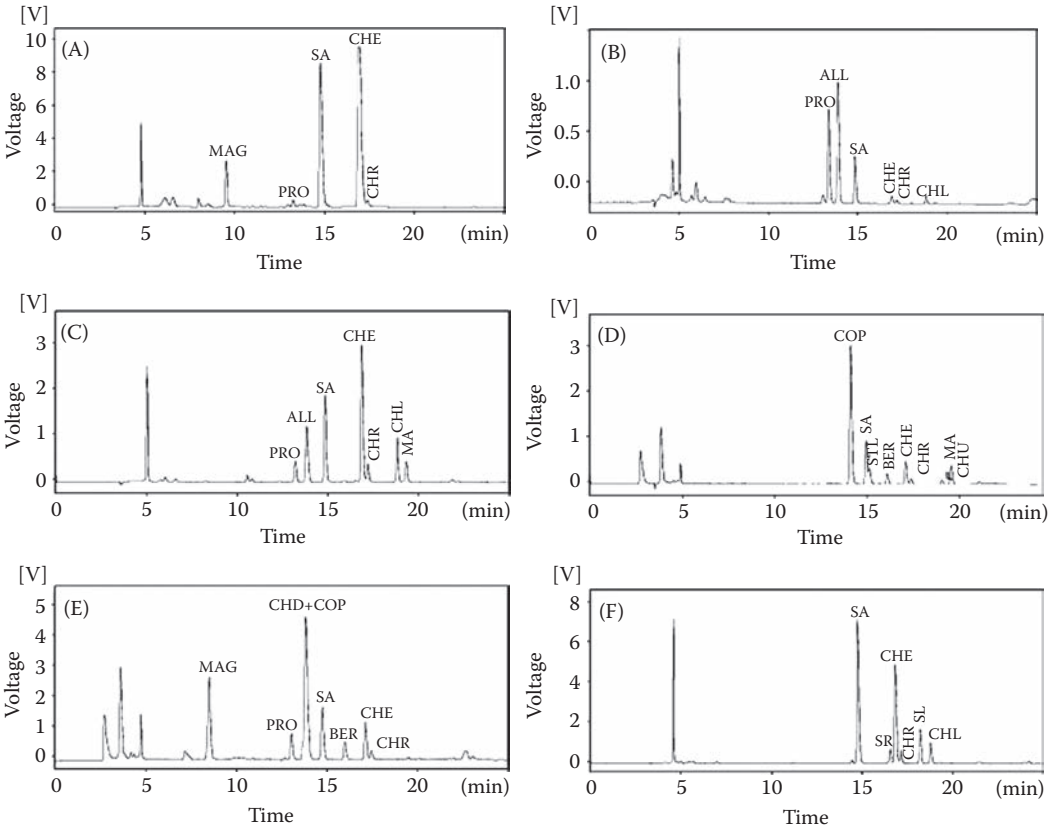

FIGURE 30.5 Representative HPLC chromatograms of extracts from the roots of (A) *D. lactucoides*, (B) *M. cordata*, (C) *M. microcarpa*, (D) *S. lasiocarpum*, (E) *C. majus*, and (F) *S. canadensis*. ALL, allocryptopine; BER, berberine; CHD, chelidonine; CHE, chelerythrine; CHL, chelilutine; CHR, chelirubine; COP, coptisine; MA, macarpine; MAG, magnoflorine; PRO, protopine; SA, sanguinarine; SL, sanguilutine; SR, sanguirubine; STL, stylopine. Conditions: analytical column, Synergi Max-RP 80A (4 μm, 150 × 4.60 mm ID); mobile phase, heptanesulfonic acid (0.01 M) and triethylamine (0.1 M) in redistilled water, pH 2.5 (H_3PO_4); the gradient was 25%–60% acetonitrile during 25 min; flow rate, 0.5 mL/min; injection volume, 50 μL; concentration of samples, 0.5 g/200 mL. (Reprinted from Suchomelová, J., Bochořáková, H., Paulová, H., Musil, P., and Táborská, E., *J. Pharm. Biomed. Anal.*, 44, 283–287, 2007. With permission from Elsevier Science.)

An HPLC-ESI-MS method was developed by Luo et al. [74] for analysis of protoberberine, indolequinoline, and quinolone alkaloids in a formula of *Coptidis rhizoma* and *Evodiae fructus* and their Chinese herbal preparations (Table 30.4). Alkaloids were separated on a Spherigel C_{18} column (5 μm; 250 × 4.6 mm ID) employing a linear gradient of ACN, MeOH, and a buffer solution consisting of 50 mM ammonium acetate and 2% acetic acid, pH 3.65. A former ion-pair RP-HPLC method [59] was modified by eliminating SDS, replacing sodium acetate with ammonium acetate, and reoptimizing gradient conditions. By comparison of on-line UV and MS information with authentic standards or literature data, 18 alkaloids were identified. The contents of five alkaloids, including berberine, palmitine, and jatrorrhizine, were determined using pseudomolecule ion and selective ion recording (SIR). The LODs for these compounds were in the range of 9–30 pg.

A rapid and sensitive HPLC-ESI-MS method using tetrahydropalmatine as an external standard was developed by Luo et al. [75] to determine sanguinarine and chelerythrine in exogenously contamined honey after their extraction into aqueous hydrochloric acid (Table 30.4). Chromatographic separations were achieved on a Spherigel C_8 (150 × 4.6 mm ID) analytical column at 30°C with

TABLE 30.4
HPLC-Mass Spectrometric Methods for Analysis of Protoberberine Alkaloids and Derivatives

Alkaloids	Isoquinoline alkaloids Californidine Chelilutine Escholamidine Chelirubine Homochelidonine Protopine	Allocryptopine Chelerythrine Escholtzine Sanguinarine *O*-methylcaryachine Glaucine *N*-methylaurotetamine Caryachine	Sanguinarine (SA) Chelerythrine (CHE)	Protoberberine alkaloids Berberine Palmatine Jatrorrhizine Coptisine Columbanine Epiberberine	Protoberberine Indolequinoline and quinolone alkaloids
Aims	*Eschscholtzia californica* Direct characterization of isoquinoline alkaloids		Exogenously contaminated honey Determination of SA and CHE	*Coptis chinensis*, *Phellodendron amurense*, *Berberis poiretii*, *B. amurensis* Chemical characterization and identification of alkaloids	*Coptidis rhizome* *Evodiae fructus* Identification and determination of alkaloids
Reference	**73. Fabre et al. 2000**		**75. Luo et al. 2004**	**77. Wu et al. 2005**	**74. Luo et al. 2005**
Sample preparation **Extraction**	5 g of dried material was extracted with 100 mL methanol (15 min., 60°C), then evaporated to dryness under reduced pressure at 35–40°C. The residue was dissolved in 50 mL water acidified to pH 1 with concentrated HCl with dodecylsulfate sodium salt (0.2%). The dodecylquaternary ammonium salts were then extracted with chloroform (3 × 50 mL). The combined chloroform extracts were dried over anhydrous Na_2SO_4 and concentrated under vacuum at 35–40°C. The residue was diluted in 10 mL of methanol and filtered before use.		Honey was mixed for 2 min with aqueous hydrochloric acid (pH 1) and extracted at 280 W for 5 min in a Galanz microwave oven. The solutions were adjusted to pH 9 with ammonia, transferred to a separating funnel, and diethyl ether was added — repeated twice. Evaporated to dryness under reduced pressure at 30°C. The residue was dissolve in 8 mL aqueous hydrochloric acid (pH 1) and the solution spiked with 50 µL internal standard tetrahydropalmatine (14 µg mL^{-1}), and then diluted to 10 mL. Portions of this solution were filtered through a 0.45 µm filter before injection.	Drugs were powdered and extracted with 75% ethanol three times. Extracts were combined and passed through a 0.45 µm filter before injection.	0.2 g sample was extracted with 70% methanol (20 mL) by sonicating (15 min), then centrifuging (5 min). Extraction was repeated once. The supernatant was diluted with 70% methanol and filtered through a 0.45 µm filter prior to LC-MS analysis.

Column	Hypersil C_8	Spherigel C_8	Dikma Diamonsil C_{18}	Spherigel C_{18}
L × ID (mm)	5 µm 150 × 4.6	150 × 4.6 5 µm Temperature 30°C	5 µm 250 × 4.6 Temperature 23°C	5 µm 250 × 4.6 Temperature 30°C
HPLC mobile phases	A: acetonitrile B: 1 mM sodium dodecylsulphate and 10 mM triethylamine in water, pH 2.5 with phosphoric acid Gradient: 0–40 min, linear gradient 20%–40% B; 40–45 min, 40% B; 45–50 min, linear gradient 40%–100% B; 50–55 min, 100% B; 55–60 min, return to 20% B	Acetonitrile—acetate buffer containing 50 mM ammonium acetate + 2% acetic acid (40:60, v/v)	A: water with 0.0034 mol L^{-1} ammonium acetate and 0.2 % acetic acid (v/v) B: acetonitrile. Linear gradient: 0–60 min, 30%–88% B	A: acetate buffer consisting of 50 mM ammonium acetate and 2% acetic acid, pH 3.65 B: acetonitrile C: methanol Gradient elution
Flow rate (mL/min)	1	1	0.5	1
Detection	ESI-MS/MS UV: 280 nm	ESI-MS (SIR) UV: 210–400 nm	ESI-FT-ICR-MS^n, ESI-MS^n UV: 277 nm	ESI-MS (SIR) UV: 250–270 nm
Limit of detection (ng)	—	SA: 1.60 CHE: 1.11	—	9–30 pg
Alkaloids	Isoquinoline alkaloids Berberrubine Coptisine Sanguinarine Nitidine Chelerythrine Liriodenine 6,7,8-trimethoxy-2,3-methylenedioxybenzophenanthridine Oxyavicine Dihydrochelerythrine	Protopine Tetrahydrocolumbamine Glaucine Palmatine Berberine Dehydrocorydaline Tetrahydropalmatine Canadine Corydaline Tetrahydrocoptisine		Dihydronitidin Dihydrochelerythrine 8-Acetonyldihydronitidine 8-Acetonyldihydrochelerythrine 1,3-Bis(8-dihydronitidinyl)acetone Nitdine α-Allocryptopine Edulinine Haplopine Skimmianine Liriodenine Arnottianamide
Aims	*Zanthoxylum nitidum* Identification and determination of alkaloids	*Corydalis yanhusuo (Rhizoma corydalis)* Identification and determination of alkaloids		*Zanthoxylum nitidum* Structural characterization and identification of alkaloids
Reference	**78. Liang et al. 2006**	**80. Ding et al. 2007**		**79. Cai et al. 2007**

(*Continued*)

TABLE 30.4 (CONTINUED)
HPLC-Mass Spectrometric Methods for Analysis of Protoberberine Alkaloids and Derivatives

Sample preparation Extraction	Powders were extracted with 70% methanol (twice) by refluxing for 2 h. The supernatant solution was combined, filtrated and then evaporated under vacuum. The residue was dissolved accurately into 100 mL solution of acetonitrile – 0.1 % formic acid aqueous solution (50: 50, v/v). The obtained solution was filtered through a syringe filter (0.45 μm) prior to HPLC analysis		Dried tuber of *Corydalis yanhusuo* was chopped and extracted three times by reflux with 70% ethanol for 1 h. After filtration, the extract was combined and evaporated to 10 mL by rotary evaporation at 50°C under reduced pressure to yield the → crude extract. The pH of the solution was set to 2 with HCl and filtered. Then the pH value of the filtrate was raised to 12 with NaOH and extracted with ethyl acetate. The extract was collected and evaporated to dryness at 50°C to obtain the → ethyl acetate extract. The ethyl acetate extract was then dissolved in 10 mL methanol. The crude extract and the ethyl acetate extract were filtered through 0.45 μm membrane filter before LC–MS analysis.		Dried roots were ground and extracted with 95% ethanol. The solvent was evaporated *in vacuo* to give an oily mass that was dissolved in 2% HCl, which was extracted with ethyl acetate, and then adjusted to pH 10 using NH_4OH to afford a precipitate. After filtration, the remaining aqueous phase was further extracted with ethyl acetate to afford the crude alkaloids used in the study.	
Column L x ID (mm)	Agilent C_{18} 5 μm 250 × 4.6 Temperature 25°C		Diamonsil C_{18} with precolumn 5 μm 200 × 4.6 Temperature 25°C		Guard column: C_{18}, ODS, 5 μm, 4 × 3 Hypersil BDS C_{18} 5 μm 250 × 4.6	
HPLC mobile phases	A: formate buffer, consisting of 1% formic acid, adjusted to pH 4.5 with ammonia (MS) / triethylamine (UV) B: acetonitrile Linear gradient: 0–80 min, 20% to 80% B (v/v)		A: 0.2 % acetic acid solution, adjusted with triethylamine to pH 5.0 B: acetonitrile Gradient: 0–15 min, 20% B; 15–35 min, linear increase to 80% B; 35–37 min, return to 20% B		A: acetonitrile B: water containing 0.5% acetic acid and 0.1% triethylamine Gradient (A:B): 25:75 (t = 0 min), 25:75 (t = 15 min), 100:0 (t = 80 min)	
Flow rate (mL/min)	1		1		1	
Detection (λ) (nm)	280		ESI-MS/MS (MRM) UV: 280 nm		ESI-MS^n ($n \leq 5$) UV: 240 nm	
Limit of detection (ng)	Berberrubine: 1.2 Coptisine: 1.5 Sanguinarine: 1.3 Nitidine: 0.6 Chelerythrine: 0.7 Liriodenine: 1.7	6,7,8-trimethoxy-2,3-methylenedioxybenzo-phenanthridine: 2.1 Oxyavicine: 1.2 Dihydrochelerythrine: 0.8	Protopine: 0.10 Glaucine: 0.10 Palmatine: 0.08 Berberine: 0.10 Canadine: 0.05 Corydaline: 0.10 Tetrahydrocolumbamine: 0.10	Dehydrocorydaline: 0.08 Tetrahydropalmatine: 0.04 Tetrahydrocoptisine: 0.05	—	

Note: ESI, electrospray ionization; FT-ICR, Fourier transform ion cyclotron resonance; MRM, multiple reaction monitoring; MS, mass spectrometry; MSn, multi-stage mass spectrometry; MS/MS, tandem mass spectrometry; SIR, selective ion recording.

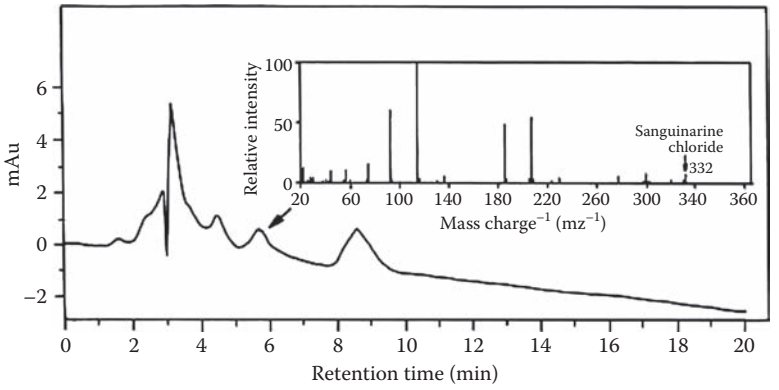

FIGURE 30.6 Typical chromatogram of an adulterated mustard oil collected from a vendor. Peak 1 = sanguinarine found 8.2 μg/g. The inset shows the mass spectrum of sanguinarine chloride. (Reprinted from Husain, S., Narsimha, R., and Rao, R.N., *J. Chromatogr. A*, 863, 123–126, 1999. With permission from Elsevier Science.)

40:60 (v/v) ACN–acetate buffer (50 mM ammonium acetate and 2% acetic acid in water). Alkaloid contents were measured by pseudomolecule ion in the SIR mode at *m/z* 332.5, 348.5, and 356.5 for sanguinarine, chelerythrine, and the internal standard tetrahydropalmitine, respectively. LODs were 1.60 ng mL^{-1} for sanguinarine and 1.11 ng mL^{-1} for chelerythrine at an injection volume of 20 μL and were three orders of magnitude lower than those reported by Husain et al. [63] (Figure 30.6) and Ševčík et al. [76].

ESI–Fourier transform ion cyclotron resonance (FT-ICR)–MS using the sustained off-resonance irradiation (SORI)/CID method and HPLC–ESI–multi-stage mass spectrometry (MS^n) were applied by Wu et al. [77] for the structural analyses of protoberberine alkaloids in *Coptis chinensis*, *Phellodendron amurense*, *Berberis poiretii*, and *B. amurensis* (Table 30.4). Standards of berberine, palmatine, jatrorrhizine, and coptisine were first analyzed by using ESI-FT-ICR-MS^n at high resolution. Then, the structures were proposed for each fragment ions obtained according their precise mass/charge ratios, and a logical fragmentation pathway of protoberberine alkaloids was proposed. Alkaloid standards were analyzed by HPLC-ESI-MS^n also to determine those ions that can be obtained by both methods and are specific and useful for identification. HPLC-ESI-MS^n was then applied for the analysis of crude extracts of medicinal herbs on a Dikma Dimonsil C_{18} column (5 μm; 250 × 4.6 mm ID) with gradient elution using ACN and water with 0.0034 M ammonium acetate and 0.2% acetic acid as the eluent. Six alkaloids were identified based on their fragmentation behavior, retention time, and mass spectra in comparison with the data from the reference standards or literature. Selected ion monitoring (SIM) was used for differentiation of isomer alkaloids.

HPLC-DAD-ESI-MS/MS was applied by Liang et al. [78] for validation of nine alkaloids from the root of *Zanthoxylum nitidum*, using authentic standards of berberubine, coptisine, sanguinarine nitidine, chelerythrine, liriodenine, 6,7,8-trimethoxy-2,3-methylendioxybenzophenanthridine, oxyavicine, and dihydrichelerythrine (Table 30.4). Quantification of these alkaloids in a 70% methanol extract of *Z. nitidum* was performed by a novel and sensitive HPLC-UV method after separation on an Agilent C_{18} column (5 μm; 250 × 4.6 mm ID) with gradient elution using ACN and 0.1% formic acid buffered to pH 4.5 with TEA. Intraday and interday reproducibilities (RSD) of amounts of nine alkaloids were less than 1.69%, and method recoveries (ranging from 98.3 to 101.1%) were acceptable. The LODs of these alkaloids ranged from 0.6 ng to 1.5 ng at 280 nm. Alkaloid contents ranged from 0.03 mg g^{-1} to 3.34 mg g^{-1} depending on the origin of the plant material, demonstrating the need for quality control of the traditional Chinese medicine *Z. nitidum*.

The fragmentation behavior and the corresponding fragmentation decomposition mechanism of six benzo[*c*]phenanthridine alkaloids, dihydrochelerythrine, dihydronitidine, 8-acetonyldihydrochelerythrine, 8-acetonyldihydronitidine, nitidine and 1,3-bis(8-dihydronitidinyl)-acetone were studied in detail by Cai et al. [79] using positive-ion ESI-MS^n. Furthermore, the crude alkaloid extract from the roots of *Z. nitidum* was rapidly analyzed by HPLC-MS^n. Alkaloids were separated on a Hypersil BDS C_{18} column (5 μm; 250 × 4.6 mm ID) using gradient elution with ACN and water containing 0.5% acetic acid and 0.1% triethylamine as solvents (Table 30.4). Ten constituents were identified by comparing their retention times and ESI-MS^n spectra with those of the authentic standards. It was concluded that detailed structural characterization can be performed by using not only the characteristic fragments but also the characteristic abundance of the fragment ions. Positional isomers of benzo[*c*]phenanthridine alkaloids can be differentiated in this way.

An HPLC-ESI-MS/MS method and an HPLC-DAD method were developed by Ding et al. [80] for qualitative and quantitative analysis of alkaloids in the traditional Chinese medicine *Corydalis yanhusuo* (Table 30.4). Ten alkaloids were unambiguously identified by comparing experimental data for retention time (t_R) and UV and MS spectra with those of authentic compounds, which are protopine, glaucine, corydaline, tetrahydropalmatine, tetrahydrocoptisine, and tetrahydrocolumbamine, as well as canadine, palmatine, berberine, and dehydrocorydaline. Furthermore, the fragmentation behavior of different types of alkaloids was studied by positive-ion ESI-MS/MS. Tertiary alkaloids give [M + H] + ions, which undergo the Retro-Diels-Alder (RDA) fragmentation reaction, as was observed for tetrahydrocolumbamine, tetrahydropalmatine, canadine, corydaline, and tetrahydrocoptisine. Since there was a double bond in the C-ring, which was opposite the B-ring, the C-ring was opened. Two fragment ions were obtained from the part of tetrahydroisoquinoline and the part of benzene ring, respectively (Figure 30.7). Quaternary alkaloids gave [M] + ions, and fragments involving losses of H, CH_3, CO, H_2O, and OCH_3 were observed only in the MS/MS spectra. Quantification of the 10 alkaloids in methanol and ethyl acetate extracts of *C. yanhusuo* was performed by the HPLC-DAD method. Separations were achieved within 35 min on a Diamonsil C_{18} column (5 μm; 200 × 4.6 mm ID) using linear gradient elution with ACN and 0.2% acetic acid solution adjusted with TEA to pH 5.0 (Figure 30.8). The LOD was less than 0.10 μg per injection. The overall intra- and interday variations were less than 5%, and the overall recovery was more than 93%, indicating the good reproducibility and accuracy of the method proposed for the quality control of *C. yanhusuo* preparations.

A simple and accurate HPLC-DAD-ESI-MS method was proposed by Chen et al. [81] for the analysis of alkaloids in *Macleaya cordata*. The analytical conditions were investigated in detail to obtain better separation and peak shape than by a formerly used method [82]. Protopine, allocryptopine, sanguinarine, and chelerythrine were used as model components in these experiments. For optimization of separation, five columns with different carbon loading (%) were

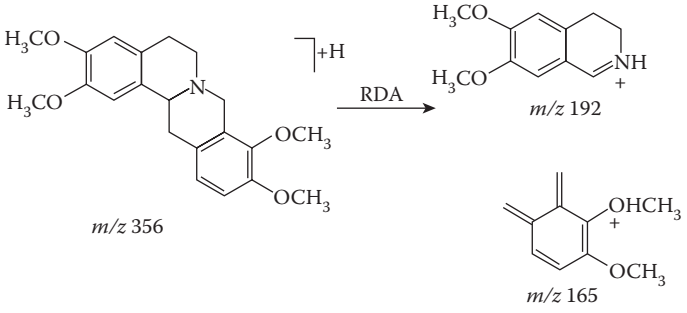

FIGURE 30.7 Proposed Retro-Diels-Alder (RDA) pathway for tetrahydropalmitine. (Reprinted from Ding, B., Zhou, T., Fan, G., Hong, Z., and Wu, Y., *J. Pharm. Biomed. Anal.*, 45, 219–226. 2007. With permission from Elsevier Science.)

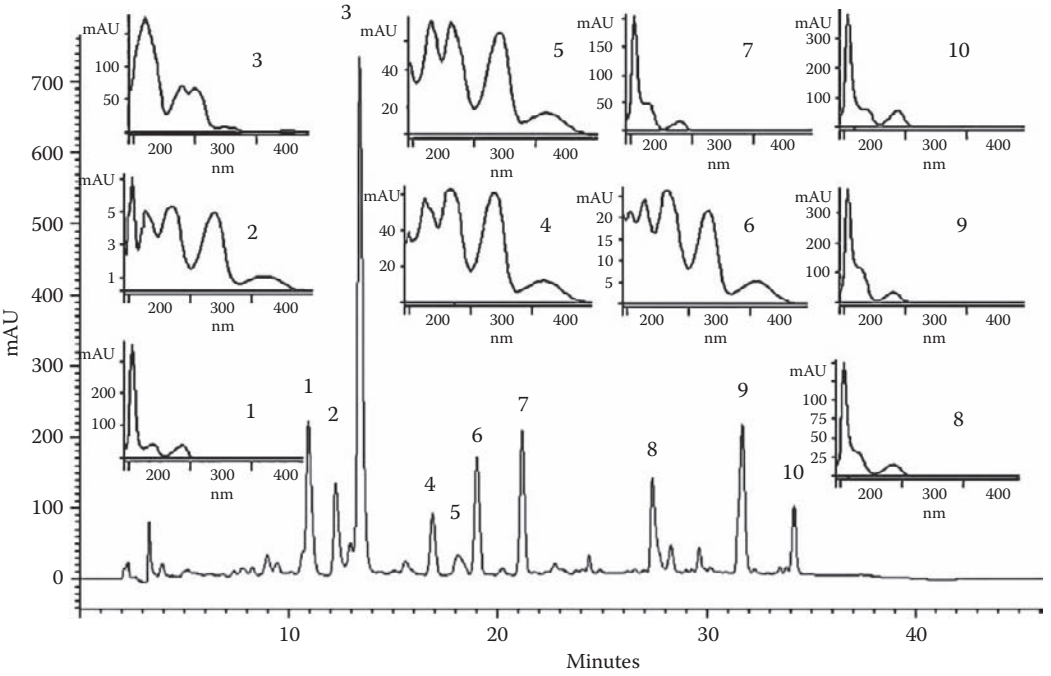

FIGURE 30.8 HPLC-DAD chromatogram of extract of *Corydalis yanhusuo* (gradient elution); diode-array UV detection (λ = 280 nm). For chromatographic conditions see Table 30.4. (Reprinted from Ding, B., Zhou, T., Fan, G., Hong, Z., and Wu, Y.,*J. Pharm. Biomed. Anal.*, 45, 219–226, 2007. With permission from Elsevier Science.)

tested: Hypersyl DBS C_{18} (11%), Spherigel C_{18} (12.5%), Ultimate XB C_{18} (17%), Kromasil C_{18} (19%), and Shim-pack VP-ODS C_{18} (20%); see Figure 30.9. Different mobile phases for the signal intensities of alkaloids in ESI-MS were investigated as well. The optimal conditions for separation and ESI-MS detection were obtained on the VP-ODS C_{18} column (with 30 mM formic acid in the mobile phase) for the analysis of alkaloids (Figure 30.10). The good performance observed for only the VP-ODS C_{18} column, when using formic acid as a mobile-phase additive, was explained by the high-carbon loading (20%), leaving fewer free silanol groups on the silica surface for ionic interactions with the protonated alkaloids. Quantitative determination of six alkaloids was performed using UV detection (284 nm) in the concentration range of 0.05–100 μg mL^{-1}. The LOD was 1.62, 1.87, 1.79, 1.76, 1.10, and 0.94 ng mL^{-1} for sanguinarine, chelerythrine, protopine, allocryptopine, dihydrosanguinarine, and dihydrochelerythrine, respectively. The LODs with ESI-MS detection were three orders of magnitude lower than those obtained with UV detection.

30.3 CONCLUSION

HPLC separation and determination of tetrahydroprotoberberine-derived alkaloids is not an easy task because of their structural diversity and common occurrence in medicinal plants (e.g., in *Chelidonium majus*, *Sanguinaria canadensis*, *Macleaya cordata*, *Zanthoxylum nitidum*, and *Coridalis yanhusuo*) and the tendency of quaternary benzop[*c*]henanthridines to interact with the stationary phase. RP-HPLC using modern silica gel (C_8, C_{12}, C_{18}) columns is currently the most popular method for analysis, because of its high separation power allowing simultaneous determination of a variety of protoberberines and derivatives with different physicochemical properties. These columns manufactured from high-purity silica gel with a densely bonded stationary phase

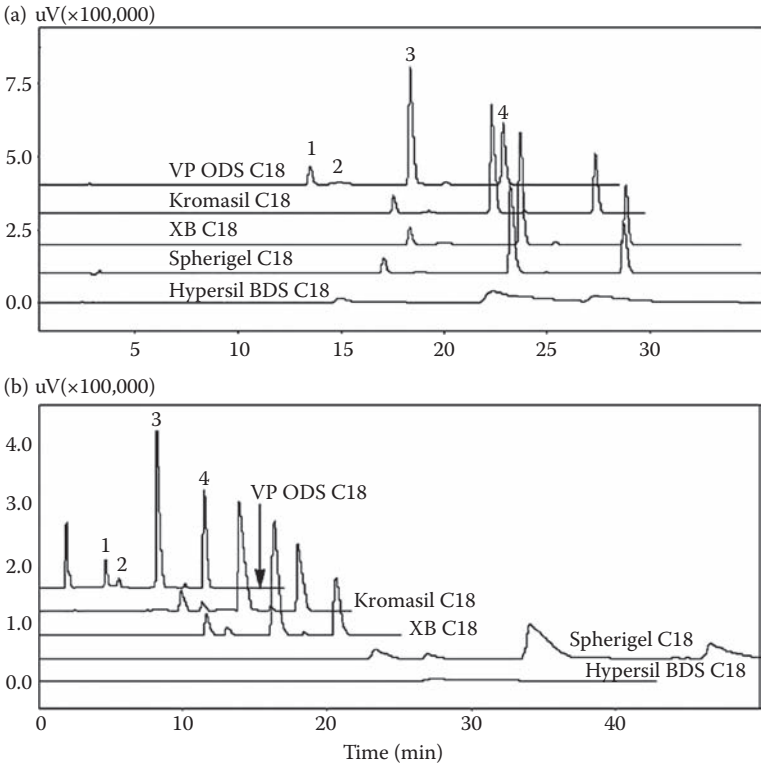

FIGURE 30.9 Effect of (a) trifluoroacetic acid (TFA) and (b) formic acid additive on the separation of the four alkaloids on five different C_{18} columns: (1) protopine; (2) allocryptopine; (3) sanguinarine; (4) chelerythrine. (Reprinted from Chen, Y.-Z., Liu, G.-Z., Shen, Y., Chen, B., and Zeng, J.-G., *J. Chromatogr. A*, 1216, 2104–2110, 2009. With permission from Elsevier Science.)

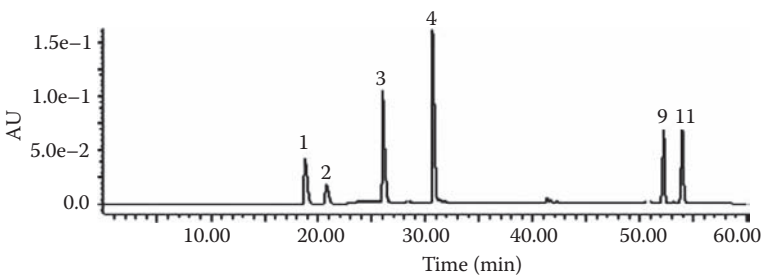

FIGURE 30.10 HPLC-UV profile of six alkaloid standards: (1) protopine; (2) allocryptopine; (3) sanguinarine; (4) chelerythrine; (9) dihydrochelerythrine; (11) dihydrosanguinarine. (Reprinted from Chen, Y.-Z., Liu, G.-Z., Shen, Y., Chen, B., and Zeng, J.-G., *J. Chromatogr. A*, 1216, 2104–2110, 2009. With permission from Elsevier Science.)

can be used to improve peak shape and selectivity [41] and simplify mobile-phase composition [42], making it compatible with MS detection [81]. Most of these alkaloids contain strong UV-VIS chromophores; therefore, highly sensitive UV-VIS detection or DAD detection can be applied, or, alternatively, more selective and sensitive fluorometric detection can be used, enabling simplification of the sample-preparation protocol.

HPLC-MS has been proved to be a powerful tool for rapid identification and determination of the protoberberine alkaloids and derivatives in botanical extracts and traditional Chinese medicines

because of its low detection limit, high specificity, and molecular mass information. Rapid structural characterization of isomeric benzo[*c*]phenanthridine alkaloids from *Z. nitidium* and protoberberine alkaloids in medicinal herbs can be performed by using HPLC with tandem MS^n alone and in combination with high-mass-resolution MS. Linearity of around three orders of magnitude of concentration was generally obtained, with LODs of 9–30 pg for protoberberine, indolequinoline, and quinolone alkaloids in the coptis-evodia herb couple determined by HPLC-ESI-MS in SIR mode. HPLC coupled with ESI-MS using SIR enabled highly sensitive determination of sanguinarine and chelerythrine in exogenously contaminated honey, with LODs (1.6 and 11.1 ng mL^{-1}) three orders of magnitude lower than those reported for HPLC-UV and capillary electrophoresis (CE)-UV. Simultaneous use of HPLC with tandem MS for identification and HPLC-DAD for determination of protoberberine, benzop[*c*]phenanthridine, and protopine alkaloids in traditional Chinese medicinal herbs and preparations yields a powerful approach for their convenient and rapid quality control. So, it can be concluded that the HPLC analysis of isoquinoline alkaloids has nearly arrived at its mature state.

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31 HPLC of Tropane Alkaloids

Tomasz Mroczek

CONTENTS

31.1 CHEMICAL DIVERSITY OF TROPANE AND RELATED ALKALOIDS

Tropane alkaloids can be divided into two series depending on the spatial orientation of the hydroxyl group at C-3 [1]. Derivatives of tropan-3α-ol (e.g., tropanol) are by far the most common, and those of tropan-3β-ol are essentially specific to the Erythroxylaceae. They are *meso* compounds, and the two bridgehead carbons have opposite chirality. Their hydroxylated forms are esterified with different types of optically active acids [1,2]; however, if other substituents are absent, the tropanols are optically inactive. The tropanols are often hydroxylated at C-6 or C-7 or both, and sometimes 6,7-epoxidized. In Figure 31.1 the structures of typical tropanols are presented, whereas in Figure 31.2, some of the tropane alkaloids in their esterified original forms are shown.

The acids that can be found in tropane alkaloids may be aliphatic (such as acetic, butyric, isovaleric, 2-methylbutyric, tiglic, and angelic acid) or aromatic [1]. In the latter case, there are specific acids like (*S*)-(–)-tropic acid, or they may be more widely distributed in the plant kingdom, like benzoic, phenylacetic, cinnamic, apotropic, and α-truxillic acid.

A relatively new class of polydroxylated-pseudotropine alkaloid derivatives are the alkaloids called calystegines [3,4]. They have been isolated from various plants of the Convolvulaceae like *Calystegia sepium* and *Convolvulus* species but thereafter were determined also in other Solanaceae plants, such as underground parts of *Atropa belladonna* and *Solanum tuberosum*. Typical structures of calystegines are shown in Figure 31.1.

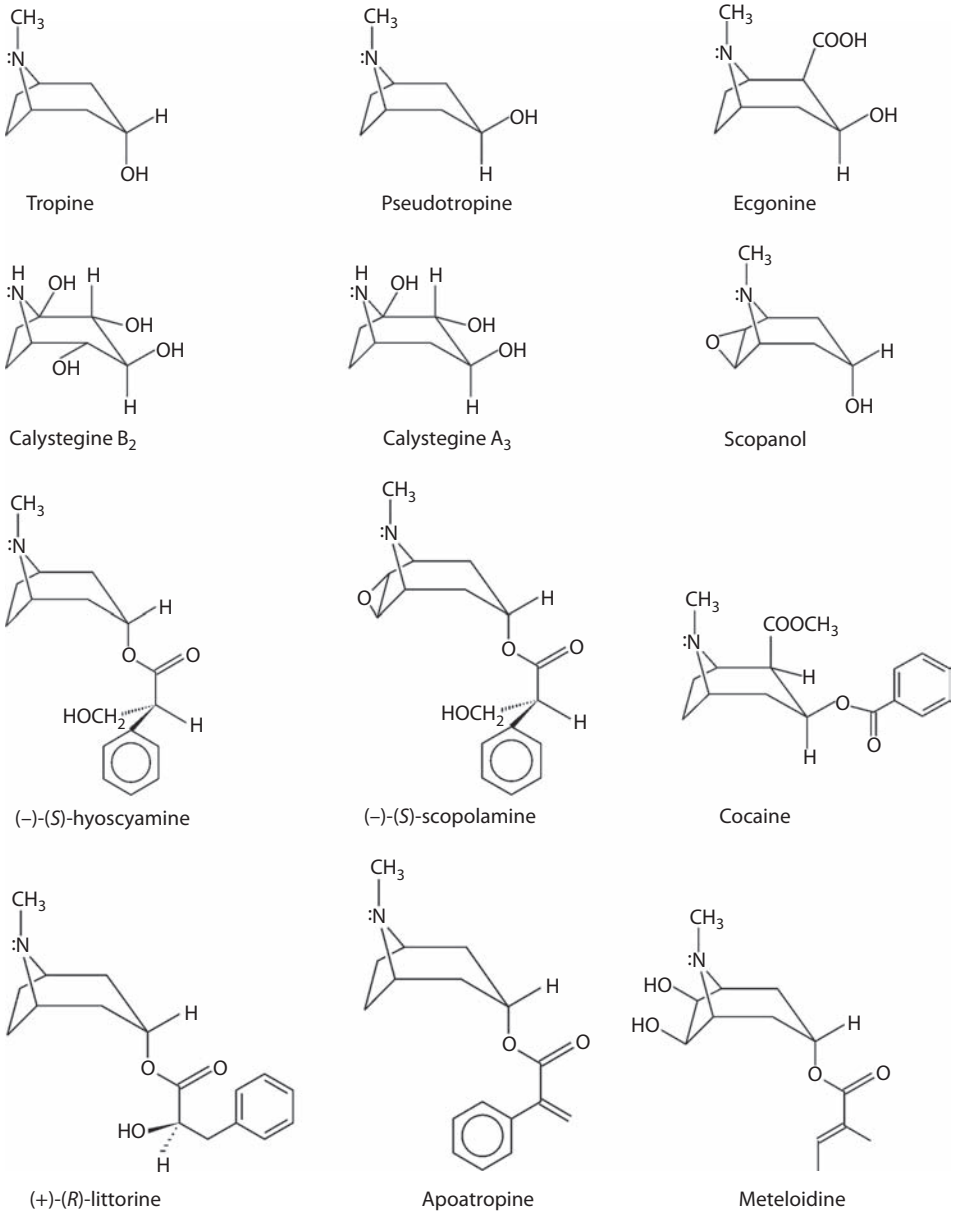

FIGURE 31.1 Chemical structures of selected tropane alcohols and alkaloids. (From Zanolari, B., Wolfender, J.-L., Guilet, D., Marston, A., Queiroz, E.F., Paulo, M.Q., and Hostettmann, K., *J. Chromatogr. A*, 1020, 75, 2003. With permission from Elsevier.)

31.2 BIOSYNTHESIS OF TROPANE ALKALOIDS

In the biosynthesis of tropane alkaloids several precursors are involved [1,5]: (a) Ornithine is at the origin of the pyrrolidine ring of the tropane nucleus; (b) phenylalanine is at the origin of the C6-C1 and C6-C3 aromatic acids as well as of tropic acid; (c) isoleucine is the precursor of C5 aliphatic acids such as tiglic acid or 2-methyl-butanoic acid; and (d) acetate (in the form of acetoacetyl coenzyme A or malonyl coenzyme A) contributes the additional carbon atoms needed to build the piperidine ring of the tropane nucleus.

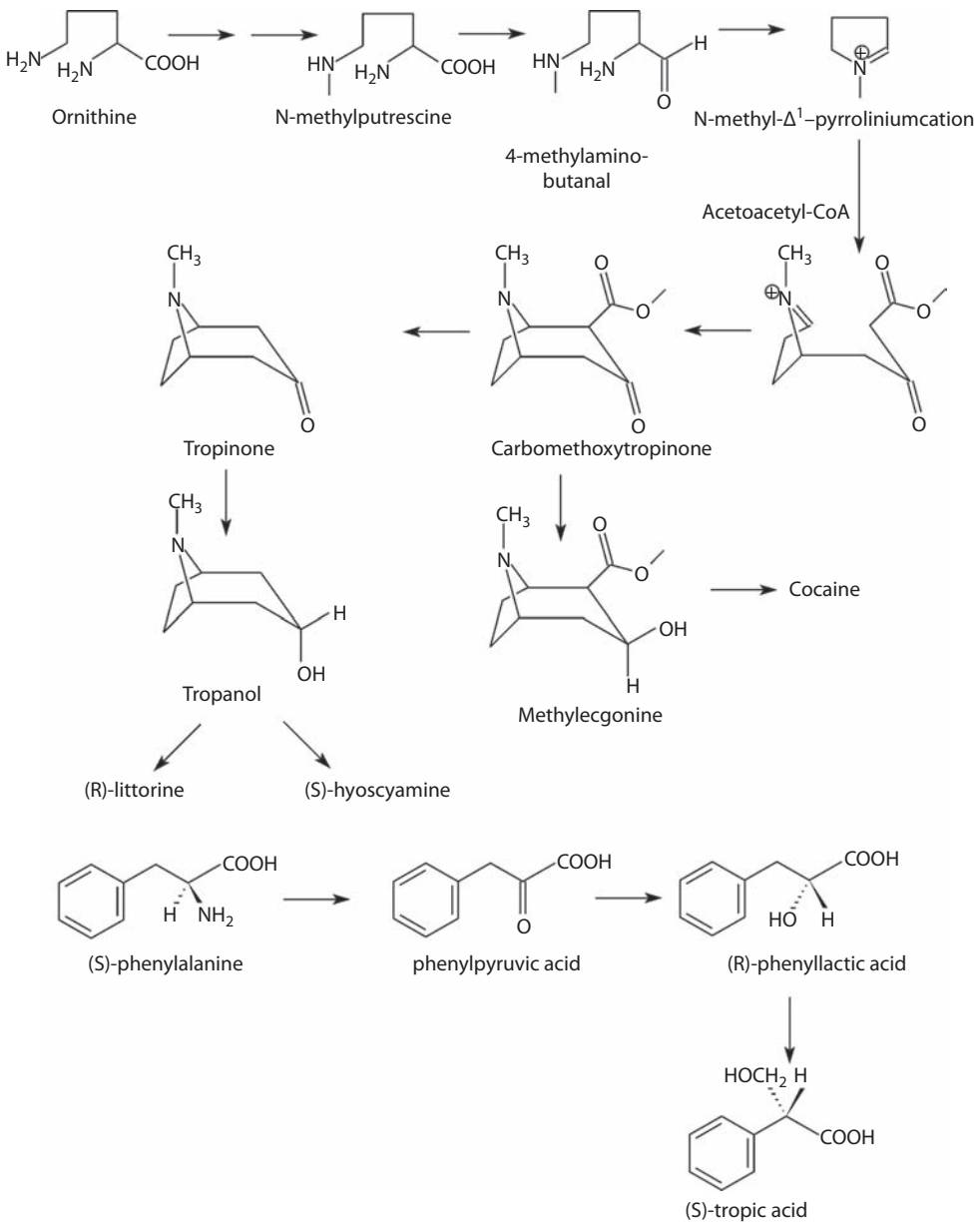

FIGURE 31.2 Biosynthesis of tropane alkaloid bases and tropic acids. (From Zanolari, B., Wolfender, J.-L., Guilet, D., Marston, A., Queiroz, E.F., Paulo, M.Q., and Hostettmann, K., *J. Chromatogr. A*, 1020, 75, 2003. With permission from Elsevier.)

Figure 31.2 shows the postulated biosynthetic pathways of the tropane alkaloid nucleus and tropic acids. At first, ornithine, the precursor of the tropane nucleus, is rapidly decarboxylated to putrescine, which is then methylated. On the other hand, putrescine can also form from arginine by decarboxylation followed by the transformation of the guanidine system into amidine and final hydrolysis of *N*-carbamoyl-putrescine. After the oxidative deamination of *N*-methylputrescine, 4-methylaminobutanal is created, which is subsequently cyclized to the *N*-methyl-Δ^1-pyrrolinium cation. This cation is considered the precursor of both the nicotine and pyrrolidine alkaloids [5]. It then condenses with acetoacetyl-CoA. After methylation of the free carboxylate group followed by

ring closure, reduction of the ketone group and benzylation results in the formation of cocaine. The esterification with (–)-tropic acid leads to (–)-hyoscyamine, whereas the reaction with (+)-tropic acid results in (+)-littorine formation. In case of hyoscyamine or littorine formation the intermediate carbomethoxytropinon is decarboxylated to tropanone, which is then reduced to tropanol. For cocaine, the carbomethoxytropanone is reduced stereospecifically to methylecgonine. The oxidation of the tropanols at C-6 and C-7 takes place after the esterification of the secondary alcohol at C-3; the epoxidation at C-6, C-7 (e.g., scopolamine) involves a 6β-hydroxykated intermediate and the direct attack of the c-6 hydroxyl group on C-7 [1]. In the biosynthesis of the tropic acids, (*S*)-phenylalanine is involved. Phenylpyruvic and phenyllactic acids are major intermediates, which lead to formation of (*R*)- or (*S*)-tropic acids.

31.3 SOLANACEAE PLANTS CONTAINING TROPANE ALKALOIDS

Solanaceae plants are a rich natural source of tropane alkaloids. They are encountered mainly in the following plant species: *Datura* sp., *Hyoscyamus* sp., *Atropa* sp., *Duboisia* sp., *Brugmansia* sp., *Mandragora* sp., *Przewalskia* sp., and so on [1]; however, only few of them are actually used in therapeutics. Besides in the Solanaceae family, these compounds have been found in Erythroxylaceae, Convolvulaceae, Protaceae, and Rhizophoraceae [5]. Here, some of the most common tropane alkaloid-containing plants are briefly described.

Deadly nightshade, *Atropa belladonna* L. Both leaves and roots contain hyoscyamine, its racemate atropine, scopolamine (the total amount of the alkaloids is between 0.3 and 0.6%), apoatropine, and aposcopolamine (their dehydration products of atropine and scopolamine, respectively) hygrine, cuscohygrine, aromatic and aliphatic amines, coumarins, phenolic acids, and tannins.

Thorn apple, *Datura stramonium* L. The leaves contain pharmaceutically active scopolamine and hyoscyamine (0.2–0.5% total). The alkaloids are also present in the seeds and may be swallowed by children, which can be very harmful. Various cultivars differ in their qualitative and quantitative composition of scopolamine and hyoscyamine [2].

Henbane, *Hyoscyamus niger* L. It is a perennial plant that was used by witches in past times. Tales of Sabbath and of levitation suggested hallucinations due to tropane alkaloids like hyoscyamine and scopolamine (0.04–0.15%).

The leaves the preceding three plant species have a foul odor due to the presence of tetramethylputrescine [1].

Coca, *Erythroxylum coca*, Erythroxylaceae. It is an illicit drug. The story of coca began in 1885, when a U.S. pharmacist J.S. Pemberton concocted a "French wine of coca, ideal tonic." He soon modified his formula, replacing the alcohol with cola extract and the plain water with fizzy water: Coca-Cola was born [1]. Coca is a cultivated shrub in Bolivia, Peru, and Argentina. The alkaloid concentration ranges from 0.5 to 1.5% depending on the species, geographical origin, and so on. Alkaloids include cocaine, cinnamylcocaine, truxillines, hygrine, and cuscohygrine.

Some Solanaceae plants are industrial sources of tropane alkaloids. They include *Brugmansia snaguinea* Ruiz & Pavon, *Datura metel* L., and *Datura innoxia* Mill as the sources of scopolamine [1], or *Hyoscyamus muticus* L. and *Duboisia myoporoides* R.Br. with high amounts of hyoscyamine.

31.4 EXTRACTION FROM PLANT MATERIAL AND SAMPLE-PRETREATMENT METHODS

31.4.1 Solid–Liquid Extraction

Due to these alkaloids' thermal instability and sensitivity to strong acidic and basic conditions, the method of solid–liquid extraction should be carefully selected. After the first step of extraction with diluted acids like 5% HCl, 0.01% H_2SO_4, or 5–10% acetic acid [6–8], usually some basic

coextractives are present in the extracts. Jia et al. [9] found the extraction of *Datura* alkaloids to be most efficient at pH 2–3. When alkaloids' free bases are to be extracted, alkaline organic phases are used.

Prior to extraction the sample is dropped with mainly 10% (or sometimes 25%) ammonia solution followed by organic solvent addition, that is, chloroform, benzene (toxic and carcinogenic, so nowadays not recommended), toluene, dichloromethane, or ethanol [6,10–12].

Fliniaux et al. [13] compared the efficiencies of both acidic and alkaline solutions for the extraction of tropane alkaloids from plant material. They used 0.2 M sulphuric VI acid, methanol–0.1 M HCl (24:1, v/v), methanol–27% ammonia (24:1, v/v), and methanol–chloroform–27% ammonia (24:1:1, v/v/v). Similar results were obtained. Calystegines are usually extracted with methanol–water mixtures as they possess hydrophilic properties [3].

Mroczek et al. [2] analyzed the content of l-hyoscyamine and scopolamine extracted from thorn apple leaves. When 1% tartaric acid in methanol was used at 90 ± 5°C on a heating mantle for 15 min, the highest amounts of scopolamine were measured, even in comparison to more sophisticated methods such as ultrasound-assisted extraction (UAE) or pressurized liquid extraction (PLE). However, the amounts of l-hyoscyamine were comparable to UAE at 60°C and lower than PLE procedures. This shows that sometimes a suitable extraction procedure should be elaborated for a given alkaloid.

With the UAE method, when methanol and 1% tartaric acid in methanol were used at room temperature, 40°C, or 60°C, the higher the temperature, the higher the yield of l-hyoscyamine and scopolamine that was measured [2]. Again, 1% tartaric acid in methanol was a better solvent than pure methanol.

For cocaine and benzoylecgonine extraction from coca leaves, focused microwave-assisted extraction (FMAE) was optimized with respect to the nature of the extracting solvent, the particle size distribution, the moisture of the sample, the applied microwave power, and radiation time [14]. FMAE generated extracts similar to those obtained by conventional solid–liquid extraction but in a more efficient manner, that is, 30 s was sufficient to extract cocaine quantitatively from leaves.

31.4.2 Pressurized Liquid Extraction (PLE)

PLE uses an organic solvent at high pressures and temperatures above the boiling point. It works according to the principle of static extraction with superheated liquids. It ensures the higher solubility of analytes in solvents at higher temperatures, a higher-diffusion rate as a result of higher temperature, and disruption of the strong solute–matrix interaction caused by van der Waals forces, hydrogen bonding, and dipole–dipole attractions between solute molecules and active sites on the matrix. This method was used to extract cocaine and benzoylecgonine from coca leaves [15].

Mroczek et al. [2] optimized PLE conditions for extraction of l-hyoscyamine and scopolamine from thorn apple leaves. In PLE experiments with methanol, similarly to the result for the ambient-pressure UAE method, the highest levels of l-hyoscyamine were measured at the highest temperature conditions (110°C) and increased considerably with increasing temperature. Therefore, the PLE procedure with methanol at default conditions turned out to be the most efficient method of l-hyoscyamine extraction from plant materials. It was slightly better than PLE with 1% tartaric acid in methanol at these conditions. In the case of scopolamine extraction the same tendency as for l-hyoscyamine was observed in PLE method but differently from this compound, the yields of scopolamine were lower when measured after rapid extraction (15 min) at 90°C with 1% tartaric acid in methanol on a heating mantle.

31.4.3 Liquid–Liquid Partitioning (LLP)

Obtained extracts contain many coextractive compounds that may interfere with the tropane alkaloids to be analyzed. Therefore, in some cases at first the acidic extract is extracted with

chloroform in a separation funnel to remove acidic coextractives. Then, the remaining extract is alkalized with ammonia solution to a pH of about 9–9.5 and extracted with nonpolar organic solvents such as chloroform, benzene, toluene, and dichloromethane [6,10–11]. However, liquid–liquid partitioning (LLP) often uses large volumes of costly solvents and is also not amenable to automation because several disjointed steps are usually required. It is also limited to small-sample volumes and solutes with large distribution constants.

31.4.4 Solid-Assisted Liquid–Liquid Partitioning (SELLP)

SELLP replaces the separation funnel and permits routine LLP to be performed simply and efficiently using a single step. It consists of a specially processed, wide-pore kieselguhr with a high-pore volume, chemically stable within pH 1–13. In the process an aqueous sample is applied to the dry column filled with granular Extrelut support. It spreads over the chemically inert matrix as a thin layer and functions as a stationary phase. Elution is carried out using organic solvents that are immiscible in water. As the solvent passes through the column, all lipophilic compounds are extracted from the aqueous phase into the organic phase. The main advantages of this method include the total lack of emulsions; savings of solvent, material, and time; simple and proper performance; and higher recoveries and cleaner eluates compared to typical LLP.

El-Shazly et al. [16] applied the SELLP procedure to the isolation of pure alkaloidal fractions from *Hyoscyamus* sp. The basified aqueous extract was applied into a dry Extrelut column, and the liquid was completely absorbed by the kieselguhr. Tropane alkaloid free bases that are exposed on the surface of the kieselguhr particles are eluted by organic solvents such as chloroform.

31.4.5 Solid-Phase Extraction (SPE)

The SPE method involves selective extraction of the analytes from liquid samples onto different varieties and types of solid supports (e.g., silica gel, alumina, florisil, kieselguhr, and reversed-phase sorbents such as octyl, octadecyl, diol, cyano, amino, and ion-exchange sorbents). The sample is directly passed through a previously conditioned cartridge filled with a given sorbent (in some new types of sorbents—so called mixed-mode or hydrophilic–lipophilic balance (HLB)—the conditioning step can be omitted), and analytes are directly collected, whereas coextractives are retained on the sorbent. It can be performed also in the reverse way when a sample is applied in a solvent of low elution strength. The analytes adsorbed are then eluted with a solvent of higher elution power.

SPE of tropane alkaloids has usually been performed on RP-18 columns. The procedure was applied for plant extracts as well as blood serum, urine, and egg yolk samples [3,17–18]. Molecularly imprinted polymer (MIP) as a selective sorbent in SPE of scopolamine from human urine and serum was also investigated [19]. However, low recoveries (46–79%) of extracted tropane alkaloids were measured. Keiner and Dräger [20] applied cation-exchange SPE for isolation of calystegines from plant samples, where they were retained by the charge of the secondary amino group.

Mroczek et al. [2] optimized a mixed-mode reversed-phase cation-exchange SPE procedure for simultaneous recoveries of l-hyoscyamine, scopolamine, and scopolamine-*N*-oxide from plant extracts as well as the quaternary alkaloid representative scopolamine-*N*-methyl bromide. Use of Oasis MCX cartridges' bimodal mechanism of retention (cation-exchange and RP) was considered for efficient purification of different types of alkaloids (tertiary bases, *N*-oxides) from plant samples as well as quaternary alkaloids potentially used as therapeutic drugs. First, three alkaloids (l-hyoscyamine, scopolamine, scopolamine-*N*-oxide) were efficiently eluted (recoveries of 80–100%) from the

Oasis MCX cartridge with methanol–10% ammonia (3:1, v/v) solution, whereas for the quaternary salt tetrahydrofuran–methanol–25% ammonia (6:1:3, v/v) was used, with recoveries of 52–76%. The authors found this SPE procedure useful for further high performance thin layer chromatography (HPTLC)-densitometric and reversed-phase high performance liquid chromatography (RP-HPLC) investigations.

31.5 HPLC OF TROPANE AND RELATED ALKALOIDS

31.5.1 Optimization of the Separation

The following tropane alkaloids have generally been taken into account in the optimization of the separation: l-hyoscyamine, atropine, apoatropine, scopolamine, their acidic and basic degradation products (like dl-tropic acid, belladonine, atropic acid, iscotropic acid), anisodine, anisodamine, 6β-hydroxyhyoscyamine, littorine, homatropine, methylhomatropine, scopolamine-*N*-oxide, scopolamine-*N*-methyl bromide, cocaine, benzoylecgonine, and calystegines A4, B4, and C1. The separation systems used and results obtained in HPLC analysis of selected tropane alkaloids have been presented in Table 31.1. In the 1970s, the first HPLC methods were proposed; these comprised a stainless steel column packed with Partisil, silver halide–impregnated silica gel, reversed-phase (RP) systems, or cation-exchange columns. To improve peak shape, ion-pair HPLC methods were broadly applied with heptanesulfonic acid, cetyltrimethylammonium bromide, and sodium dodecyl sulfate (SDS) used as the counterions. Next, RP systems were developed with phosphate buffer under acidic conditions or triethylamine under basic conditions. A combination of two different-polarity columns (RP-cyano followed by RP-18) was also proposed for atropine and scopolamine analysis in plant extracts.

The methods developed since the beginning of the 21st century started from supercritical fluid chromatography (SFC) utilizing CO_2 with polar modifiers and a cation-exchange CS3 IB column, a SIL5 amino column, or a charge-transfer XTerra C18 column. For cocaine and benzoylecgonine separation, different chromatographic systems utilizing C18, C8, palmitamidopropyl, or pentafluorophenylpropyl stationary phases were investigated. More sophisticated methods involved column switching with a MIP (molecularly imprinted polymer) precolumn and a conventional cation-exchange analytical column, or ultra performance liquid chromatography (UPLC) on a hydrophilic lipophilic chromatography (HILIC) silica column or a C18 column with a sorbent with a pore diameter of only 1.7 μm. This reduced the total time of the analysis (from about 20–40 min to about 1.5 min (calystegines) and 7 min (other tropane alkaloids). Unfortunately, the UPLC system was applied only for pure standards' mixtures. Combination of UPLC with mass spectrometry for more complex mixtures of tropane alkaloids with different polarities and stereochemistry would be very challenging.

Only a few papers have dealt with the separation of tropane alkaloids' enantiomers. For this purpose a Chiracel OD column or silica gel with covalently bonded β-cyclodextrins was proposed. Enantioseparation of atropine is also possible on achiral RP-18 material alone using (2*S*,3*S*)-dicyclohexyl tartate in phosphate buffer (pH 2.8) [50].

An important issue in tropane alkaloids' investigation by HPLC is the choice of a suitable detection system. Due to lack of sufficient chromophores, UV tracks can be recorded at only 205–215 nm. Cocaine derivatives exhibit absorbance at about 230 nm, whereas calystegines ought to be analyzed using other detectors better suited for nonchromophores, like a refraction index (RI) detector, evaporative light scattering detector (ELSD), pulsed amperometric detector, or mass spectrometric (MS) detector after electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), or thermospray ionization (TSP, a rather old-fashioned type). In the more obsolete methodologies, precolumn derivatization into chromophoric and fluorescence derivatives (with 1-antroyl cyanide giving fluorescence product) was applied.

TABLE 31.1
HPLC Separation Systems of Tropane Alkaloids Investigated

Compounds	Separation System	Results	Ref.
Atropine, scopolamine, apoatropine	Stainless steel column packed with Partisil, eluted with diethyl ether–methanol (95:5) and 1% diethylamine; UV detection	Good separation of the investigated compounds	[21]
Hyoscyamine, scopolamine, and others	Silver halide impregnated silica gel for HPLC in isocratic or simple gradient modes	Lower retention but better specificity, good chemical and physical stability of this HPLC system; applicable in routine analysis	[22]
Hyoscyamine, scopolamine, and others	Small-particle silica gel column eluted with ion-pair reagent (trinitrobenzenesulfonic acid) added to the mobile phase; UV detection at 215 nm	Up to 50-fold enhancement in detection limits for several tropane alkaloids; the reproducibility was below 2% (relative standard detection)	[23]
Atropine sulfate and scopolamine hydrobromide in tablets	Ion-pair HPLC; UV detection at 230 nm	Good quantitation system, reproducibility below 1%, detection limit below 0.5 μg/injection	[24]
Hyoscyamine, scopolamine, and others	Cation-exchange HPLC followed by postcolumn derivatization using the fluorimetric ion-pair technique	Improved sensitivity and selectivity for the determination of tropane alkaloids	[25]
Atropine, scopolamine, and others in eye drops together with chlorhexidine acetate	Reversed-phase C8 column eluted with methanol–water (5:5) with 5 mM heptanesulfonic acid sodium salt at pH 3.5; UV detection	Reproducible procedure for pharmaceutical form of tropane alkaloid–containing eye drops	[26]
Atropine and its acidic and basic degradation products	Ion-pair reversed-phase HPLC: Lichrosorb RP-8 eluted with citrate-phosphate buffer (3 parts, pH 5.6) and 2.7 mM cetyltrimethylammonium bromide (2 parts) in methanol; UV detection at 254 and 430 nm	Nine compounds including belladonine, apoatropine, tropic acid, atropic acid and isotropic acid were identified in 1% aqueous sulfate irradiated with UV for 24 h	[27]
Atropine and scopolamine in *Datura* sp. and *Atropa belladonna*	Diethyl ether extracts were separated on Porasil B and MicroPak Si-5 columns using tetrahydrofuran–diethylamine (100:1); refraction index detector was used	Separation and quantitation of atropine and scopolamine in different *Datura* sp. and *Atropa belladonna* roots	[28]
l-Hyoscyamine and scopolamine in *Scopolia* radix	Methanol–water (7:3) extracts were examined using Nucleosil 5C18 stationary phase and 50 mM aq. KH_2PO_4 (pH 3.5) containing 1% triethylamine–acetonitrile (85:15) as the mobile phase	Quantitative determination of scopolamine and l-hyoscyamine in *Scopolia* radix collected at 10 different places	[29]
Enantiomers of tropane alkaloids and synthetic derivatives	Silica gel with covalently bonded β-cyclodextrin	Racemic atropine, oxyphenonium, andsome of the aromatic carboxylic acids obtained by hydrolysis of tropane alkaloids or synthetic derivatives could be separated; application of muscarinic receptors is also discussed.	[30]

Atropine, hyoscyamine, and scopolamine in Solanaceae species	Ion-pair HPLC with μBondapak C18 column and mobile phase consisting of methanol–water (7:13 v/v) with heptanesulfonic acid (5mM), and phenylpropanolamine as an internal standard; UV detection at 254 nm	Rapid, sensitive, and facile method of the screening in Solanaceae species	[31]
Hyoscyamine, scopolamine, and others	μBondapak C18, 10 μm column eluted with methanol–acetic acid–triethylamine–water mobile phases; UV detection	Improved peak shape, capacity factor, and theoretical plates number	[32]
Hyoscyamine and scopolamine in plant cell cultures	A polymeric RP column with alkaline ammonium acetate buffer–acetonitrile mobile phase; selected ion recording of the protonated molecular ions was used for quantitation; the compounds were fragmented by discharge-assisted ionization and elevated thermospray capillary temperatures or ion-repeller potentials	Good linearity in quantitative determination as well as good-quality MS spectra of the alkaloids	[33]
Hyoscyamine (atropine), anisodine, anisodamine, and scopolamine	Ion-pair HPLC combined with column switching for determination of tropane alkaloids in complex preparations; the tropane alkaloid fraction immediately eluted by the primary mobile phase without SDS from a pretreatment column was transferred to an analytical column and separated by the ion-pair mobile phase	Linear calibration was obtained in the range of 0.3–30 μg/mL for anisodine, anisodamine, and scopolamine, and 0.3–300 μg/mL for hyoscyamine (atropine); recovery about 97%	[34]
Atropine and scopolamine in plants	Combination of two different-polarity columns (RP-cyano followed by RP-18) in series and direct injection of plant extracts	A simple and rapid HPLC method of screening various solanaceous plants	[35]
Hyoscyamine, scopolamine, anisodamine, and anisodine	Micellar HPLC with SDS added to the mobile phase; the mobile phase was optimized with the modified simplex method	Simple, sensitive, and accurate method without need of sample preparation	[36]
Scopolamine and hyoscyamine in feeds and biological samples	After solid-phase extraction on C18 cartridges, RP-HPLC on Lichrosorb RP-18 with mobile phase consisting of acetonitrile–methanol–0.05 M ammonium acetate (20.9:27.9:51.2) in isocratic mode, flow rate 1.3 mL/min; internal standard: bamifylline; UV detection at 210 nm	Simple and accurate method with limits of detection: 12 ng and 13.25 ng respectively for scopolamine and hyoscyamine determination	[37]
l-hyoscyamine, l-scopolamine, dl-tropic acid in plant material	RP-HPLC separation using acidic aqueous acetonitrile; UV detection at 204 nm	The absolute detection limits are 20 ng for the alkaloids and 5 ng for tropic acid; simple and convenient method	[13]
Atropine and scopolamine in *Scopolia* extracts	HPLC with precolumn derivatization with 1-anthroyl cyanide in acetone; fluorescence detection using a RP column: excitation wavelength of 255 nm and an emission wavelength of 474 nm	Good linearity and limit of detection at 0.01 μg/mL of the alkaloid.	[38]
6β-Hydroxyhyoscyamine, scopolamine, hyoscyamine, and littorine in hairy roots of *Atropa*	ODS-120 A column, kept at 40°C and eluted isocratically with acetonitrile–10 mM SDS (pH 3.3); UV detection at 215 nm	Good quantitation tool	[39]

(*Continued*)

TABLE 31.1 (CONTINUED)
HPLC Separation Systems of Tropane Alkaloids Investigated

Compounds	Separation System	Results	Ref.
Atropine, scopolamine, homatropine, methylhomatropine, tropic acid, enantiomers of the alkaloids	RP-HPLC using Hypersil-5 ODS column and eluted with methanol–aq. phosphate buffer and 0.01 M tetrabutylammonium bromide; the chiral separation was performed on Chiracel OD (Daicel) column with mobile phases: *n*-hexane–2-propanol–methanol–triethylamine (80:10:10:0.0125 or 85:5:10:0.025, v/v/v/v); UV detection	Selective method for the compendial purity control; moderate sensitivity (50 ng); separation of *S* and *R* enantiomers of hyoscyamine	[40]
Hyoscyamine and atropine in *Atropa belladonna*	Supercritical fluid chromatography (SFC) utilizing carbon dioxide and methanol, trifluoroacetic acid, or triethylamine as solvent systems	Good sensitivity with below 1 ng of hyoscyamine per signal	[41]
Calystegines in plant material and other structurally similar polydroxylated alkaloids	HPLC systems with a pulsed amperometric or MS detector with thermo- or electrospray ionization. Columns: Dionex CS3 cation-exchange (SCX), IB-SIL5 amino, or similar; elution systems: 10 mM hydrochloric acid (in SCX column), acetonitrile–water (5:95) with 15 mM ammonium formate	Baseline separation on ion-exchange column within 20 min; limit of detection from 10 to 50 ng/mL; structural differentiation by MS detection system	[42]
Tropane alkaloids in *Erythroxylum vacciniifolium*	LC-APCI-DAD-MS-NMR analysis using Nucleodur 100-5C18 column with precolumn, eluted with acetonitrile–water gradient with additional amounts of trifluoroacetic acid; DAD, TOF-MS, ion trap MS, and NMR detection	24 alkaloids were determined on-line including 6 new natural compounds	[43]
Hyoscyamine, scopolamine, and apoatropine in *Atropa belladonna* cultures	HPLC separation on Luna C8 column and isocratic mobile phase consisting of acetonitrile–30mM phosphate buffer–methanol (12.2:79.7:8.1, v/v/v); DAD detection at 210 nm	Simple and sensitive method with good separation efficiency; quantitative analysis	[44]
Atropine and its degradation products	A Thermo Hypersil Aquasil C18 column eluted with 20 mM phosphate buffer (pH 2.5) and acetonitrile (gradient mode); UV detection at 215 nm	Robust and simple procedure, which may replace ion chromatography in the study of atropine and its degradation products; fully validated	[45]
Atropine and scopolamine in *Scopolia* extract	HPLC with column switching: a MIP (molecularly imprinted polymer) precolumn and a conventional cation-exchange analytical column – eluted with phosphate buffer (pH 6.0)–acetonitrile mobile phase; temp. 40°C; UV detection at 210 nm	Rather complicated but accurate and reproducible procedure	[46]
Hyoscyamine, scopolamine, 6β-hydroxyhyoscyamine, and apoatropine in solanaceous hairy roots	Luna C18 column with precolumn and mobile phase consisting of acetonitrile–methanol–30 mM phosphate buffer, pH 6.0 (12:7.9:80.1); DAD detection at 210 nm	A simple and reproducible method without ion-pair reagents	[18]

l-hyoscyamine, scopolamine, scopolamine-*N*-oxide, and scopolamine-*N*-methyl bromide in *Datura* sp.	XTerra C18, eluted with acetonitrile–water with 15 mM ammonia water solution (gradient procedure); DAD detection at 205 nm	Fast, efficient, reproducible, and sensitive procedure for screening of tropane alkaloids in various *Datura* sp.; absolute limit of detection about 5 ng, without ion-pair reagent additive	[2]
Isomeric tropane alkaloids from *Schizanthus grahamii*	HPLC-UV-MS/SPE-NMR using a cryogenic flow probe and HPLC-NMR with loop storage; columns: Hypercarb porous graphitic carbon (PGC) eluted with acetonitrile–D_2O with 0.1% formic acid (loop storage method, 60 µL – the volume of NMR flow cell), and water–methanol with 0.1% formic acid (UV, MS detection); SPE-NMR with HySphere GP resin cartridges (d_p 10–12 µm) after postcolumn addition of 0.1% aq. formic acid; trapped compounds were then subsequently flushed with 250 µL of deuterated acetonitrile or deuterated methanol into the NMR flow cell (30 µL volume).	Increased sensitivity for the determination of four isomeric alkaloids on-line in the extracts; good-quality COSY spectra could be also recorded	[47]
Cocaine, benzoylecgonine, and lidocaine as internal standard	Different chromatographic columns: C18, C8, palmitamidopropyl, pentafluorophenylpropyl: phosphate buffer (pH 3)–acetonitrile; UV detection at 230 nm.	RP-amide turned out to be the best chromatographic system	[48]
Calystegines A4, B4, C1, scopolamine, 6β-hydroxyhyoscyamine, hyoscyamine, littorine	Ultra performance liquid chromatography (UPLC): isocratic hydrophilic–lipophilic chromatography (HILIC) – Acquity column (d_p 1.7 µm) eluted with 95% methanol–5% aq. ammonium acetate buffer (10 mM), pH 5 (calystegines); Acquity BEH C18 (d_p 1.7 µm) columns eluted with 15% acetonitrile–aq. buffer with 0.1% trifluoroacetic acid; evaporative light scattering detector (ELSD)	Rapid separation within 1.5 min (calystegines) and 7 min (other tropane alkaloids); symmetrical peaks of the alkaloids	[49]

Note: APCI, atmospheric-pressure chemical ionization; COSY, correlation spectroscopy; DAD, diode-array detection; LC, liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate; SPE, solid-phase extraction; TOF-MS, time-of-flight mass spectrometry.

31.5.1.1 Quantitative Analysis and Method Validation

Ion-pair HPLC methods showed good reproducibility (relative standard deviation [RSD] < 1%), with a limit of detection (LOD) below 0.5 μg/injection of atropine and scopolamine. Linear calibration curves with ion-pair HPLC using SDS as the counterion were obtained in the range of 0.3–30 μg/mL for anisodine, anisodamine, and scopolamine, and 0.3–300 μg/mL for hyoscyamine or atropine. Recoveries were about 97%. Good linearity and a low LOD (0.01 μg/mL) were achieved by HPLC with precolumn derivatization giving a fluorescence product with 1-antroyl cyanide. Methods with RP sorbents under acidic or basic conditions gave absolute LODs in the range of 12–20 ng for scopolamine and hyoscyamine, 50 ng in the case of chiral separation of *S* and *R* enantiomers of hyoscyamine, or even as low as 5 ng when the charge-transfer sorbent XTerra C18 and a diode array detector (DAD) were applied. Lower LOD values were measured only for the SFC-APCI-MS method (about 0.7 ng for atropine).

Examples of the quantitative analyses of different tropane alkaloids involve plant extracts, pharmaceutical dosage forms, standards mixtures, or root cultures. According to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines, the method specificity, precision (including intra- and interday precision), accuracy, linearity, and sensitivity are determined for full validation of the quantitative data [2,34,41,44].

31.5.2 Hyphenated Techniques

Hyphenated techniques play an important role in tropane alkaloid analysis. First, as was mentioned before, they are necessary because of the lack of chromophores. Second, they facilitate direct structural determination of the individual constituents present in minute amounts in the extracts. Thus, they allow discovery of new natural tropane alkaloids in the plant species from Solanaceae or Erythroxylaceae.

31.5.2.1 Liquid Chromatography–Mass Spectrometry (LC-MS)

The first approach developed by Auriola et al. [33] in 1991 utilizes a polymeric RP column with an alkaline buffer–acetonitrile mobile phase and discharge-assisted ionization and elevated thermospray capillary temperatures or ion-repeller potentials. Selected ion recording (SIM) of the protonated molecular ions was used for quantitation, and good-quality MS spectra could be recorded.

Considerable improvement in the sensitivity was achieved by application of more modern ionization systems like APCI or ESI. A method described by Dost and Davidson [41] in 2000, using SFC-APCI-MS with CO_2–15% MeOH containing 0.5% trifluoroacetic acid (TFA), enabled determination of atropine with sensitivities below 1 ng. Unfortunately, it has not been applied for plant extracts containing other tropane alkaloids.

Two further LC-MS hyphenated techniques that were developed by Zanolari et al. [43] in 2003 and Bieri et al. [47] in 2006 show the extended possibilities of HPLC-hyphenated techniques in determination of potentially new tropane alkaloids in the extracts. They also utilized HPLC–nuclear magnetic resonance (NMR) for complete structure assignment, which is explained later. In Zanolari et al.'s method both DAD and APCI-MS were used, while Bieri et al. used a high-resolution MS detector (TOF, or time of flight), which facilitates accurate mass measurements of the novel alkaloids (with mass error about 10 ppm), and an ion trap (IT) MS, which was used for determination of fragmentation patterns. In Figures 31.3 and 31.4, HPLC-UV-APCI-MS analysis of the crude alkaloid extract from *Erythroxylum vacciniifolium* and the proposed fragmentation pattern for a novel compound with *m/z* 430 are respectively presented.

The work by Bieri et al. [47] exemplifies the fascinating possibility of HPLC-hyphenated methodology in the screening of *Schizanthus grahamii* extract. The instrumental setup applied is presented in Figure 31.5. The HPLC-DAD-ESI-ion trap-MS compartment works simultaneously with two on-line NMR systems, which are further described in the next section.

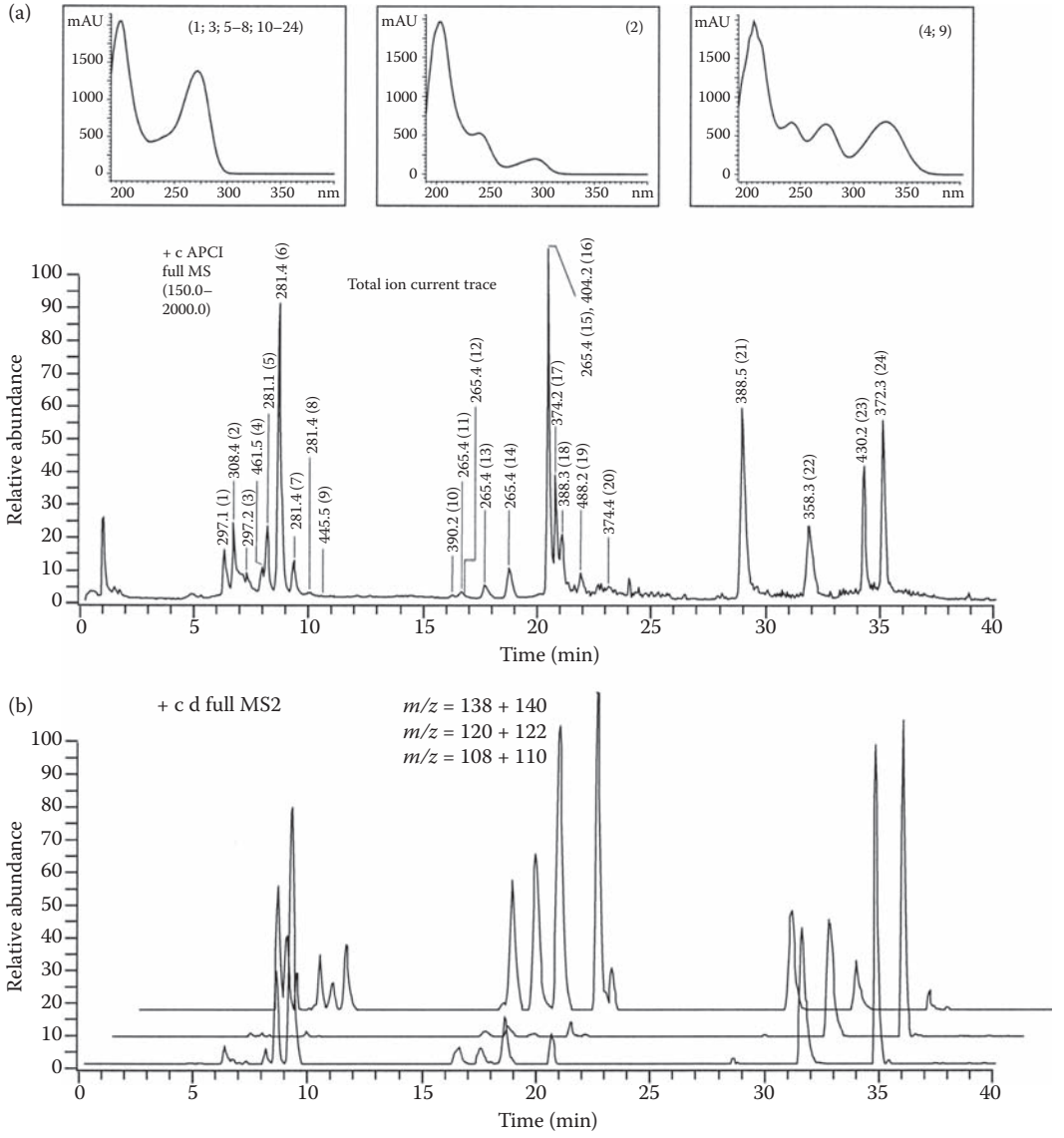

FIGURE 31.3 (A) Liquid chromatography–UV–atmospheric pressure chemical ionization–mass spectrometry analysis of the crude alkaloid extract of *E. vacciniifolium*. The total ion current (TIC) trace was recorded in the positive mode between 150 and 2000 units. The UV spectra were recorded between 200 and 500 nm. (B) Selective tandem mass spectrometry traces of characteristic fragment ions. (From Zanolari, B., Wolfender, J.-L., Guilet, D., Marston, A., Queiroz, E.F., Paulo, M.Q., and Hostettmann, K., *J. Chromatogr. A*, 1020, 75, 2003. With permission from Wiley InterScience.)

31.5.2.2 Liquid Chromatography–Nuclear Magnetic Resonance (LC-NMR)

Figure 31.6 shows on-flow (the upper picture) and stop-flow (the lower picture) LC-NMR analysis of the alkaloid extract of *Erythroxylum vacciniifolium* [43]. This is an example of powerful LC-NMR analysis of novel tropane alkaloids. In general, stop-flow spectra were more sensitively recorded than on-line ones. These two compounds were previously suggested by the HPLC-APCI-TOF (or IT)-MS method. A complete hyphenation of all these techniques together could be problematic because of the differences in the sensitivities of the detectors.

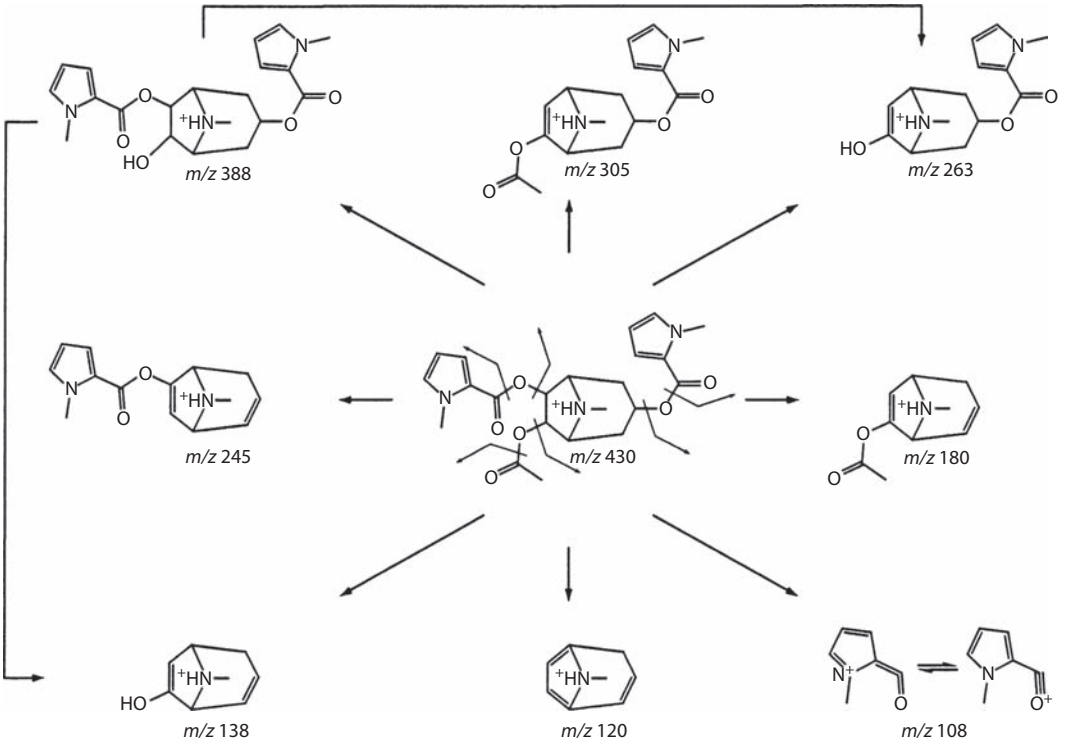

FIGURE 31.4 Proposed ion trap mass spectrometric fragmentation pattern for compound 23 isolated from *Erythroxylum vaccinifolium*. Due to the higher *M*r, compound 23 was selected as an example. The other molecules gave similar low-*M*r fragment ions. (From Zanolari, B., Wolfender, J.-L., Guilet, D., Marston, A., Queiroz, E.F., Paulo, M.Q., and Hostettmann, K., *J. Chromatogr. A*, 1020, 75, 2003. With permission from Elsevier.)

In another HPLC-NMR approach developed by Bieri et al. [47] and presented graphically in Figure 31.5, a SPE-NMR device with a cryogenic flow probe was used, and then another one with a loop storage. Trapped compounds on HySphere GP resin cartridges were subsequently flushed with a small volume of deuterated solvent (250 μL) into the NMR flow cell of 30 μL. This led to a dramatic increase in measured sensitivities. Thus, two-dimensional (2D)-NMR correlation spectroscopy (COSY) spectra could be recorded very efficiently. Such a spectrum for 3α-senecioyloxy-7β-hydroxytropane is presented in Figure 31.7. Compared with the stop-flow mode, the loop storage approach avoids interrupting the chromatographic process for NMR measurements. The insertion of SPE allows the separation to be conducted with nondeuterated solvents.

31.5.3 Comparison of HPLC and Other Chromatographic Methods (TLC, GC, CE, CZE, MECC)

Parr et al. [51] applied HPLC, gas chromatography (GC), and GC-MS methods to the transformed root cultures of the genera *Datura*, *Scopolia*, and *Hyoscyamus* for the determination of alkaloids' patterns in different species. These methods turned out to be complementary in chemotaxonomic research.

Several tropane alkaloids present in the food-poisoning sample of *Datura* roots were also studied by HPLC, GC-MS, and thin-layer chromatography (TLC) with well-correlated quantitative results for scopolamine and atropine levels [52]. GC-MS versus HPLC was also applied once more in l-hyoscyamine and scopolamine determination but in blood serum [53].

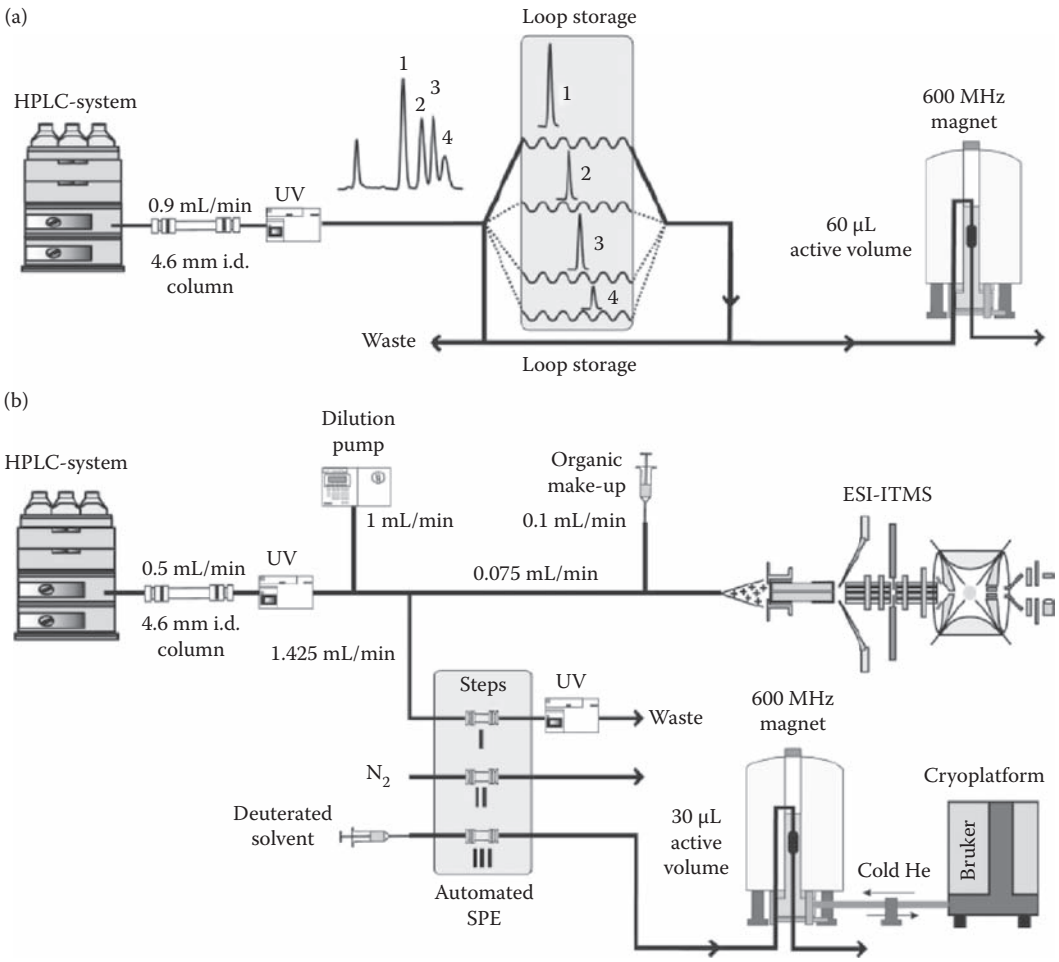

FIGURE 31.5 Instrumental setup for the HPLC–nuclear magnetic resonance hyphenated approaches for *Schizanthus grahamii* extract: (A) peak sampling unit using storage loops; and (B) peak trapping onto solid-phase extraction cartridges with parallel mass spectrometry and cryogenically cooled nuclear magnetic resonance detection. (Taken from Bieri, S., Varesio, E., Veuthey, J.-L., Munoz, O., Tseng, L.-H., Braumann, U., Spraul, M., and Christen, P., and Hostettmann, K., *Phytochem. Anal.*, 17, 78, 2006. With permission from Wiley InterScience.)

Mroczek et al. [2] compared RP-HPLC-DAD and HPTLC-densitometry of l-hyoscyamine and scopolamine determination in a broad range of *Datura* sp. samples. Good correlation between HPLC and HPTLC quantitative results was measured, although two different quantitation modes were applied. Correlation coefficients of mean values in overall analyses were 0.92086 and 0.99995 for l-hyoscyamine and scopolamine respectively. Each value correlated was in the range of $r \pm 2SD$. In terms of precision, the HPLC-DAD method turned out to be slightly more precise than HPTLC-densitometry.

Other separation techniques like capillary electrophoresis (CE), capillary zone electrophoresis (CZE) [54], or micellar electrokinetic chromatography (MEKC) [55] were mainly applied for efficient enantiomeric or quaternary alkaloids separation, utilizing, for example, sulfated β-cyclodextrins. These techniques can therefore be used instead of HPLC on chiral sorbents. On the other hand, HPLC methods are usually more sensitive than CE or CZE, and less demanding in terms of optimization of the experimental conditions.

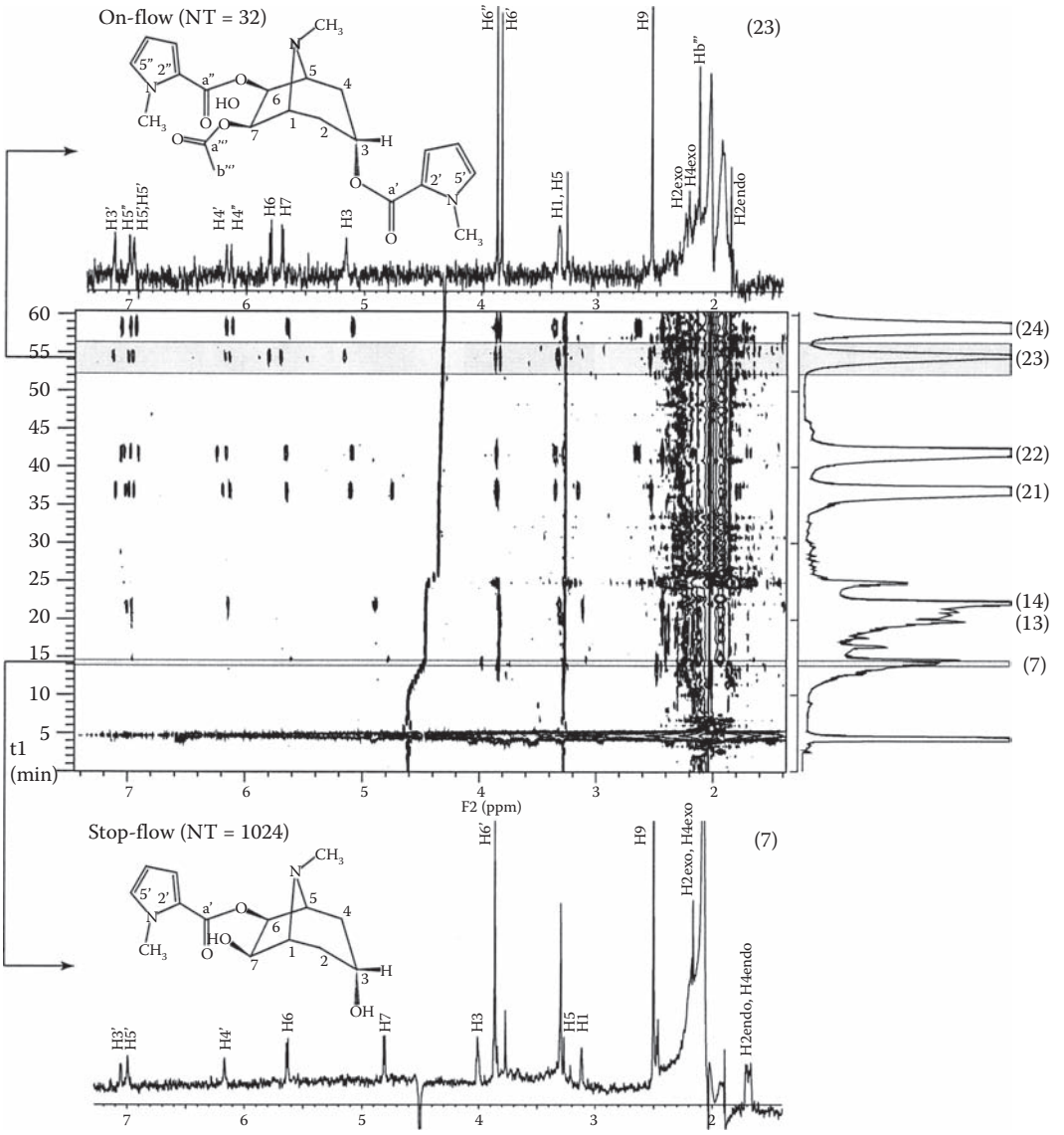

FIGURE 31.6 On-flow liquid chromatography–nuclear magnetic resonance (NMR) contour plot of the alkaloid extract of *E. vacciniifolium*. On this two-dimensional plot all ^{1}H-NMR resonances of the analytes appear as dots. ^{1}H-NMR spectra of compounds 7 and 23 are shown as examples of resolution for on-flow and stop-flow analyses, respectively. (From Zanolari, B., Wolfender, J.-L., Guilet, D., Marston, A., Queiroz, E.F., Paulo, M.Q., and Hostettmann, K., *J. Chromatogr. A*, 1020, 75, 2003. With permission from Elsevier.)

31.6 CONCLUSIONS

HPLC methods together with GC-MS play the primary role in the analysis of tropane alkaloids. For the determination of known alkaloids (mainly hyoscyamine, scopolamine, and apoatropine) HPLC-UV or HPLC-DAD are most often applied; however, sensitivities in some cases are moderate (at about 20–50 ng per alkaloid injected). To improve sensitivity and separation efficiency and reduce the analysis time, HPLC (or rather UPLC) hyphenated techniques, especially with ion trap (or TOF) MS after ESI, should be considered. For complete structural determination of novel compounds in the extracts, SPE-NMR stop-flow techniques are the most efficient, although restricted by

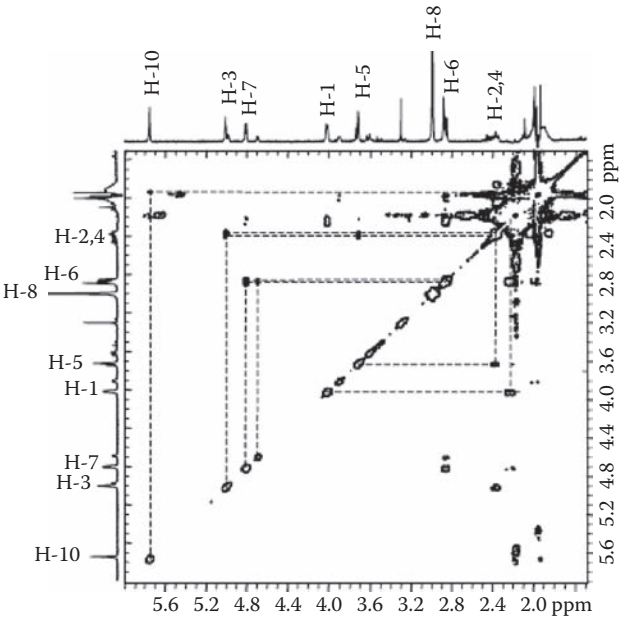

FIGURE 31.7 Correlation spectroscopy (COSY)-^{1}H-nuclear magnetic resonance (NMR) spectrum of isomer **1** (3α-senecioyloxy-7β-hydroxytropane) using the HPLC–UV–mass spectrometry–solid-phase extraction–NMR setup (Taken from Bieri, S., Varesio, E., Veuthey, J.-L., Munoz, O., Tseng, L.-H., Braumann, U., Spraul, M., and Christen, P., *Phytochem. Anal.*, 17, 78, 2006. With permission from Wiley InterScience.)

the still-high costs of such sophisticated analytical devices. In the case of separation of enantiomers, HPLC and CZE using chiral sorbents are the methods of choice.

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32 HPLC of Alkaloids from the Other Biosynthetic Groups

Jolanta Flieger

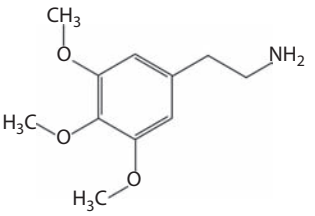
CONTENTS

32.1 PHENYLETHYLAMINE DERIVATIVES

32.1.1 Classification of Phenylethylamine Derivatives

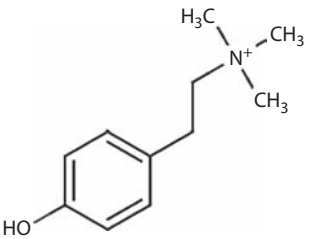
This group of alkaloids is structurally based on 2-phenylethylamine, which was isolated together with its *N*-methyl derivative from *Accacia prominent*. Many analogues of phenylethylamines are hallucinogenic substances that exist naturally but can also be produced synthetically. As regards hallucinogenic phenylethylamines, mescaline is one of greatest importance. This is the main alkaloid of the cactus *Lophophora williamsii*, better known as peyote, whose native region extends along the southwestern United States. As a classic hallucinogen, it is often used as the potency standard

against which all other phenylethylamine bases are compared. A totally synthetic analogue of mescaline is 3,4,5-trimethoxyamphetamine (TMA).



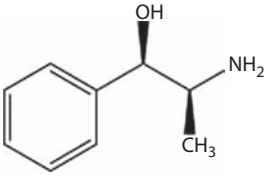
(1) Mescaline

Derivatives of phenylethylamine also occur as quaternary ammonium compounds. One of them is candicine (2), isolated from *Trichocereus candicans* cactus.

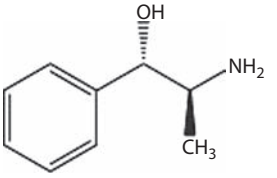


(2) Candicine

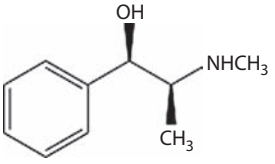
Another subgroup of phenylethylamine alkaloids is formed by ephedrine and its derivatives. Ephedrine alkaloids are the active constituents of plants belonging to the *Ephedra* genus. Ephedrine, possessing two asymmetric carbon atoms, can form four optical enantiomers that may differ in pharmacological activity as well as in the rate of their metabolism. (–)-Ephedrine (5) is a major isomer; minor alkaloids include (–)-norephedrine (3), (+)-norpseudoephedrine (4), (+)-pseudoephedrine (6), and (–)-methylephedrine (7).



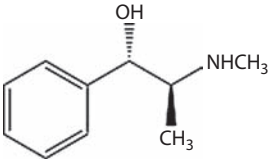
(3) (–)-Norephedrine



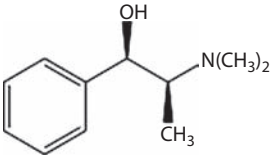
(4) (+)-Norpseudoephedrine



(5) (–)-Ephedrine

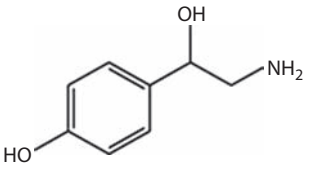


(6) (+)-Pseudoephedrine

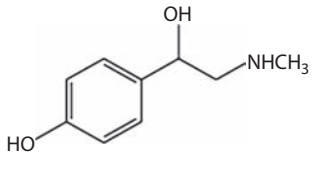


(7) (–)-Methylephedrine

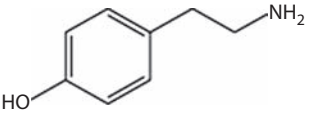
Citrus aurantium (bitter orange), belonging to the Rutaceae family, contains phenylethylamine alkaloids, mainly (±)-octopamine (8), (±)-synephrine (9), tyramine (10), *N*-methyltyramine (11), and hordenine (12). Synephrine is the main alkaloid; it is similar in structure to ephedrine. As a chiral compound, it also possesses enantiomers exhibiting different pharmacological activity toward α and β adrenoreceptors.



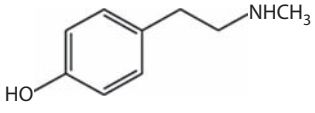
(8) (±)-Octopamine



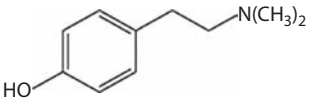
(9) (±)-Synephrine



(10) Tyramine



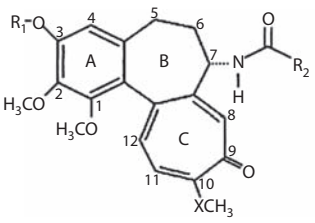
(11) *N*-Methyltyramine



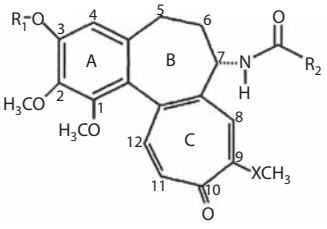
(12) Hordenine

The last subgroup belonging to phenylethylamine derivatives is the group of *Colchicum autumnale* alkaloids. The main alkaloid of this group is colchicine. It is a powerful anti-inflammatory agent. Unfortunately, it exhibits genotoxic activity by mitosis inhibition. A dose over 0.8 mg/kg usually results in cardiogenic shock. Colchicine is known to be sensitive to light and hydrolysis. The main degradation products described are β- and γ-lumicolchicine and colchiceine.

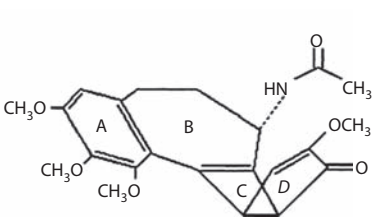
General structures of *Colchicum* alkaloids are presented below.



I
Normal form



II
iso form



III
β lumi derivative

(13) General structures of *Colchicum* alkaloids

32.1.2 Sample Preparation

Application of high performance liquid chromatography (HPLC) requires preliminary sample preparation providing a sample free of components that may deteriorate the column. Sometimes this step can also be aimed toward increasing the sample concentration or removing interfering components. In the literature, a number of methods for the analysis of alkaloids in plant materials, pharmaceutical formulations, and biological samples have been described, including liquid–liquid extraction, solid-phase extraction (SPE), and, recently, supercritical fluid extraction [1]. Exemplary methods of sample preparation are collected in Table 32.1.

TABLE 32.1
Preparation of Samples of Different Origin Containing Phenylethylamine Alkaloids

Sample Source	Alkaloid	Extraction Procedure	Ref.
Powders, resins, contents of capsules, tablets; dried plants	Mescaline, 3,4,5-trimethoxyamphetamine (TMA)	Powdered samples (each 20 mg) were extracted with 2 mL of methanol by ultrasonication for 10 min. After centrifugation (5 min at 3000 rpm), the solutions were filtered through a centrifugal filter device. All extraction procedures were performed under protection from daylight; amber glass utensils were used.	[2]
Lophophora williamsi, *Trichocereus pachanoi*	Mescaline	The pretreatment of the sample was performed by grinding the fresh cactus and extracting the jelly pulp obtained either with a methanolic–ammonia solution or an aqueous solution buffered at pH 4.0 (phosphate buffer).	[3]
Colchicum seeds	Colchicine, colchicoside	10 g of sample with 0.15 g of $CaCO_3$ was extracted with 90% methanol in Soxhlet during 6 h. After evaporation, the sample was reconstituted in 100 µL of methanol.	[4]
Kampo medicines	Ephedrine, pseudoephedrine, norephedrine, methylephedrine	A daily dosage of crude drugs was extracted with 500 mL of water for 1.5 h. The decoction was filtered while hot and the volume adjusted to 250 mL with water after cooling. The decoction was centrifuged and the supernatant was filtered.	[5]
Urine	Norephedrine, norpseudoephedrine, ephedrine, pseudoephedrine, *n*-methylephedrine, ethylephedrine	20 µL of phenylpropylamine solution (1000 µg/mL), 100 µL of 10 M sodium hydroxide, and 2 mL of diethyl ether were added to 2 mL of urine, then the urine was saturated with 1 g of sodium sulfate and shaken for 20 min. After centrifugation the organic layer was removed and evaporated to dryness in a vacuum evaporator at a temperature up to 18ºC. The residue was dissolved in 100 µL of the mobile phase.	[6]
Traditional Chinese medicinal preparations	(−)-Ephedrine, (+)-Pseudoephedrine	Powdered sample (1 g) was dissolved with 10 mL water in an ultrasonic bath for 5 min at 30ºC. Then, a 10 mL mixture solution of 4 g sodium chloride and 10 g sodium hydroxide was added and distilled. About 100 mL of the distilled solution was collected in a flask containing 50 mL 1% hydrochloric acid and concentrated to 20 mL. The pH value of solution was adjusted to 4 with 10% sodium hydroxide and concentrated to 5 mL. Then the solution was diluted with water as an HPLC sample.	[7]
Ephedra species	Ephedra alkaloids	6 mL of acetone was added to 500 mg of ground plant material. The mixture was sonicated for 15 min. After sonication the sample was centrifuged for 10 min. The supernatant was collected. The extraction was repeated two more times and the respective supernatants were combined and the acetone removed. 5 mL of absolute ethanol was added to the dried extract.	[8]
Ephedra sinica	Ephedra alkaloids	Samples (500 mg) of a product or 1 g of the finely powdered plant material were extracted three times with 3 mL 0.37% hydrochloric acid by sonication. After centrifugation the extracts were combined and filled up to 10 mL with extraction solvent. Prior to use, all samples were filtered.	[9]

Natural health products and formulations	Ephedra alkaloids	The sample was mechanically shaken in approximately 25 mL 10 mmol monobasic potassium phosphate with 3% methanol (extraction solution) for 15 min, then sonicated for 45 min. After cooling, the sample was diluted to 50 mL and well shaken with the extraction solution. The mixture was centrifuged for 20–40 min to remove all residual suspended particulate matter.	[10]
Citrus species	Phenylethylamine alkaloids: (±) octopamine, (±)-synephrine, tyramine, *N*-methyltyramine, hordenine	The sample preparation from *Citrus* plant material (fruits and peel) involved a sonication extraction of a weighed amount of the ground sample (0.5 g) with 10 mL of water at room temperature for 15 min. After centrifugation for 3 min at 4000 rpm, the supernatant solution was filtered in a vacuum into a 25-mL volumetric flask. The residue was reextracted in the same way. The filtrates of the two extractions were combined, and water was then added to the final volume. Regarding *C. aurantium*, dry extracts, and dietary supplements, a weighed amount of sample (0.1 g of dry extracts; 0.2–0.4 g of powdered tablets or the capsule contents) was extracted twice with 10 mL of water and sonication. All the extracts were filtered through a 0.45-µm cellulose acetate filter into a HPLC vial and capped. The extraction procedure was repeated twice for each sample.	[11]
Ephedra sinica Stapf	(−)-Ephedrine	A volume of 0.5 mL Chinese *Ephedra* extract was applied on the molecularly imprinted solid-phase extraction cartridge after the cartridge was conditioned with 20 mL acetonitrile. The cartridge was then eluted with 10 mL washing solvent (acetonitrile) followed by 5 mL elution solvent (5% trifluoroacetic acid in methanol). Eluted fractions were collected and dried with air stream. The residues were dissolved with 0.5 mL acetonitrile and analyzed by analytical RP-HPLC.	[12]
Human plasma	Colchicine	An aliquot of plasma (100 µl) was placed in a 10 mL glass tube followed by 100 µL I.S. solution and 3 mL *n*–hexane–dichloromethane–isopropanol (300:150:15, v/v/v). The mixture was vortex-mixed for 30 s and shaken for 10 min. After centrifugation at 3500 × *g* for 5 min, the organic phase was transferred to another 10 mL glass tube and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted in 100 µL mobile phase. Recovery: 91.9%–103.0%.	[13]

32.1.3 Chromatographic Systems for Separation of Phenylethylamine Derivatives

Among the phenylethylamine-type alkaloids, ephedrine is one of the pharmacologically most important substances. A variety of chromatographic and electrophoretic separation techniques have been used for the determination of ephedrine in plant materials and different products containing *Ephedra* species (Table 32.2).

The separation of ephedrines by HPLC is difficult due to the similar structures and basic character of these polar compounds. Because of that, they can interact strongly with free silanols on the surface, causing peak tailing and affecting either the resolution or the quantitative analysis. Therefore, the determination of ephedrines requires reversed-phase packings based on high-purity silicas with a low level of silanol activity (endcapped bonded phases) and mobile phases with high-ionic strength or amine modifiers as a mobile-phase additive [3].

Liquid chromatography of ephedrine alkaloids is most often accomplished on reversed-phase stationary phases based on alkyl-bonded silica particles with mobile phases containing ion-pairing reagents such as sodium dodecyl sulfate (SDS) [5] and related compounds [25]. Mobile-phase additives suppress silanophilic interactions and increase the retention of the phenylethylamine alkaloids. This technique was applied by Nobuyuki et al. [5] for the determination of ephedrine alkaloids in Kampo medicines containing Herba Ephedrae. Analysis was performed within 25 min by the use of ion-pair reversed-phase HPLC and isocratic elution (Figure 32.1).

It should be stressed, however, that the main limitation of ion-pair reagents used as mobile-phase additives is their poor volatility and ion-suppressing effects, making this method less amenable to mass spectrometric (MS) analysis. Gay and White recently reported a system suitable for LC-MS analysis of ephedrine alkaloids; however, the high-aqueous content of the mobile-phase employed definitely limits the method sensitivity [26]. Another disadvantage of these agents is difficulties in restoring the initial properties of the column.

In contrast to the highly hydrophobic alkyl sulfonates possessing high affinity to the hydrophobic stationary phase, less hydrophobic anionic additives (ClO_4^-, PF_6^-, CF_3COO^-) appear to be more practical for this purpose, which has been reflected in their recent popularity. According to the Hofmeister classification of salts, they are known as chaotropic salts. A very important advantage offered by these additives, besides the increase in retention, is their ability to decrease the peak width. To describe influence of chaotropic salts on the retention of basic compounds, the following terms could be used: Hofmeister effect or chaotropic effect [27,28].

Addition of ionic liquids to the mobile phase also improves the peaks' shape and separation of basic compounds. Both anionic and cationic components of added reagents contribute to solute retention owing to their salting-out and ion-pairing effects. For example, *C. aurantium* extract was analyzed on the HPLC column Spherigel C_{18} (250 × 4.6 mm ID, 5 μm) and a mobile-phase consisting of an aqueous solution of 32 mM 1-ethyl-3-methylimidazolium tetrafluoroborate EMIM BF_4 in isocratic conditions [29].

Derivatization procedures, for instance, with 9-fluorenylmethylchloroformate [20] or *o*-phthaldialdehyde *N*-acetyl-L-cysteine (NAC) [30] or changing the properties of the stationary phase into strong cation-exchange [31] were also described for separation of ephedrine and its derivatives.

As alternatives to commonly used C_{18} and C_8 phases, fluorinated stationary phases have recently been gaining acceptance owing to their unique selectivity for phenylethylamine alkaloids. The ion-exchange mechanisms are characteristic of fluorinated phases. Pellati and Benvenuti elaborated on separation conditions for ephedrine alkaloids and synephrine using a pentafluorophenylpropyl (PFPP) stationary phase and mobile-phase compatible with MS [11] (Figure 32.2). Bell et al. [19] also used a PFPP stationary phase for the separation of ephedrine alkaloids and synephrine. The strategy applied in this study allowed a complete separation of ephedrine alkaloids and synephrine within 18 min with a satisfactory peak shape.

Several HPLC methods have been reported for the determination of ephedrines in biological fluids and in pharmaceutical preparations. However, they are not always sufficiently efficient for

TABLE 32.2
HPLC Systems for Separation of Phenylethylamine Alkaloids

Aim of Separation	Compounds	Stationary Phase	Mobile Phase	Detection	Ref.
Cactaceae species	Mescaline	C_{18}-reversed-phase	5.0 mM aqueous solution of octylamine o-phosphate	230 nm	[3]
Psychotropic/ psychoactive drugs present in different products	11 tryptamines/β-carbolines and 8 phenylethylamines	Atlantis dC_{18} column	A: 10 mM ammonium formate (pH 3.5)–acetonitrile (95:5) B: Acetonitrile–methanol (7:3) Gradient mode (A:B): 100:0 (0 min) to 95:5 (15 min) to 90:10 (35 min) to 73:27 (52 min) to 30:70 (60 min) Flow rate 0.3 mL/min	PDA (monitored at UV 210 nm)	[2]
Impurity of methamphetamine	(1S,2R)-(+)-ephedrine, (1R,2S)-(−)-ephedrine, (1S,2S)-(+)-pseudoephedrine, and racemic methylephedrine	Phenyl-β-cyclodextrin column, ODS column	20 mmol/L KH_2PO_4 (pH 4.6)–CH_3CN (4:1) 5 mmol/L SDS in 20 mmol/L KH_2PO_4–CH_3CN (65:35)	210 nm	[14]
Simultaneous determination of ephedrines in urine	Norephedrine, norpseudoephedrine, ephedrine, pseudoephedrine, n-methylephedrine, ethylephedrine	LiChrospher 60 RP-Select B, LiChrospher 100 RP18, Hypersil BDS-C_{18}, Inertsil ODS-2, Spherisorb ODS-B, and Symmetry Shield RP8	50 mM phosphate buffer–25 mM triethylamine	215 nm	[6]
Chiral separation of ephedrines	Norephedrine, ephedrine, pseudoephedrine, N-methylephedrine, N-methylpseudoephedrine	Immobilized β-CD a LichroCard ChiralDex LiChrospher 100 RP18	Methanol–acetate buffer–TEA Methanol–acetate buffer–β-CD	215 nm	[15]
Traditional Chinese medicinal preparation (Jiketing Granule)	(−)-Ephedrine, (+)-pseudoephedrine	Hypersil C_{18}	Acetonitrile–0.02 M dihydro-potassium phosphate solution containing 3% triethylamine (4:96)	210 nm	[7]
Ephedra species (chemical fingerprinting)	Ephedra alkaloids	Xterra RP_{18}	A: Water B: Acetonitrile Gradient elution: 75% A and 25% B for 10 min; 0% A and 100% B over 45 min; 100% B for 10 min	320 nm	[8]

(*Continued*)

TABLE 32.2 (CONTINUED)
HPLC Systems for Separation of Phenylethylamine Alkaloids

Aim of Separation	Compounds	Stationary Phase	Mobile Phase	Detection	Ref.
Ephedra sinica	(±)-Octopamine, (±)-synephrine, tyramine, (–)-norephedrine, (+)-pseudoephedrine, and (–)-ephedrine	HyperClone C_{18} BDS 1 column (100 × 4.6 mm ID, 3 µm)	A: 3 mM aqueous SDS solution with pH 4.0 adjusted with 5% phosphoric acid (v/v) B: 0.1% aqueous phosphoric acid (v/v) containing 3 mM SDS with pH 2.0 C: Acetonitrile–methanol (2:1, v/v); Elution started with 75% A, 0% B, 25% C, changed over 10 min to 0% A, 6% B, 35% C, to reach 0% A, 60% B, 40% C. Flow rate: 1 mL/min, injection volume: 10 µL	210 nm	[9]
Dietary supplements containing *Ephedra* and *Citrus* alkaloids	Synephrine	YMC phenyl column (250 × 2.0 mm ID, 5 µm)	2% Acetic acid, 44 mM ammonium acetate, and 3% acetonitrile	APCI-MS/MS	[16]
Plant material from *Ephedra* species and *Ephedra*-containing natural products.	Ephedrine alkaloids	Discovery HS F5 column pentafluorophenylpropyl (PFPP)	Ammonium acetate (7 mM) in acetonitrile–water (90:10, v/v), under isocratic conditions	215 nm	[17]
Ephedrine alkaloids in natural health products		SynnergiPolarRP	3% Acetonitrile, 2% acetic acid, 50 mmolL ammonium acetate in water, further diluted 20-fold	210 nm	[18]
	Ephedrine alkaloids	Pentafluorophenylpropyl-bonded liquid chromatography columns (Discovery HS F5)	The pH values of the mobile phases (85% or 90% aqueous acetonitrile mixtures) were unadjusted (pH 6.7) prior to the addition of organic modifier. Ammonium acetate was added as modifier to obtain the desired molar concentration	220 or 215 nm	[19]
Plasma samples	Ephedrine and norephedrine	Symmetry 5 µm C (150 × 4.6 mm ID)	Acetonitrile and water (52:48, v/v)	264, 313 nm	[20]
Citrus aurantium plant material (fruits and peel and dietary supplements claiming to contain C. *aurantium*)	Phenethylamine alkaloids (i.e. (±)-octopamine, (±)-synephrine, tyramine, N-methyltyramine, and hordenine)	Pentafluorophenylpropyl (PFPP) stationary phase (Discovery HS F5 column)	10 mM ammonium acetate in acetonitrile–H_2O (90:10, v/v)	225 nm	[11]

Citrus and *Ephedra* in plant material (peel) and commercial products	Octopamine, synephrine, tyramine	HyperClone C_{18} BDS 1 column (100 × 4.6 mm ID, 3 µm)	3 mM aqueous SDS (pH 4.0), 0.1% (v/v) H_3PO_4 with 3 mM SDS and acetonitrile/methanol (2:1, v/v), gradient	UV, 210 nm	[9]
Human plasma	Synephrine	Zorbax SCX column (150 × 2.1 mm ID, 5 µm)	5 mM aqueous ammonium formate (pH 3.0) and acetonitrile, gradient	ESI-MS/MS	[21]
The plant material (leaves and unripe fruits of *Citrus* species)	p-Synephrine	Nova-Pak C-18 column, 150 × 3.9 mm ID, 5 µm (Waters, Milford, USA) and a C-18 guard column, 10 × 4 mm ID, 10 µm (Waters, Milford, USA)	A: Acetonitrile–water–TFA (5:95:0.01, v/v/v) B: Pure acetonitrile Gradient elution: 0–8 min, 100%–59% A; 8–10 min, 59%–0% A; 10–12 min, 0% A; 12–13 min, 0%–100% A; 13–18 min, 100% A	220 nm	[22]
Human plasma	Methylephedrine, noscapine	Diamonsil C_{18} column, 150 × 4.6 mm ID, 5 µm, with a Security Guard C_{18} (5 µm, 4 × 3.0 mm ID) guard column	Methanol–water–formic acid (70:30:0.5, v/v/v)	APCI-MS	[23]
Human plasma	Colchicine	Zorbax Extend C_{18} column, 5 µm, 150 × 4.6 mm	Formic acid–10 mM ammonium acetate–methanol (1:49:75, v/v/v)	ESI-MS	[13]
Colchicum seeds	*Colchicum* alklaloids	Chromolith RP-18e column, 100 × 4.6 mm	27% methanol and 73% (v/v) of phosphate buffer, pH 6.0	245 nm	[24]

Note: APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PDA, photodiode array detection. SDS, sodium dodecyl sulfate; TEA, Triethylamine; β-CD, β-cyclodextrin; CD, cyclodextrin; TFA, Trifluoroacetic acid.

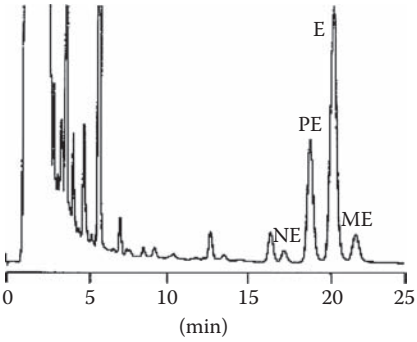


FIGURE 32.1 Chromatogram of Kakkon-to decoction. Peaks: NE, norephedrine; PE, pseudoephedrine; E, ephedrine; ME, methylephedrine. Column: Wakosil-II 5C_{18} HG. Eluent system: water–acetonitrile–sodium dodecyl sulfate (65:35:0.4). Flow rate: 1.0 mL min^{-1}. Detection: 210 nm. (From Nobuyuki, O., Hirotsugu, M., Takako, H., Satie, Y., Yasue, M., Yumi, N., Megumi, T., Hironori, Y., and Akira, Y., *J. Pharm. Biomed. Anal.*, 20, 363, 1999. With permission.)

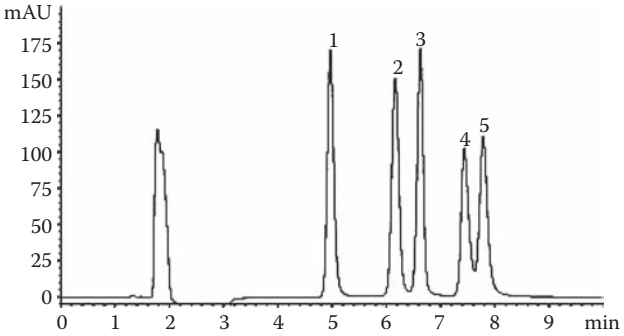


FIGURE 32.2 Chromatogram of a standard mixture of phenethylamine alkaloids. Column, Discovery HS F5 (150 × 4.6 mm ID, 5 µm). Mobile phase, 10 mM ammonium acetate in acetonitrile–water (90:10, v/v). Flow rate, 1 mL/min. Column temperature, 20°C. Sample injection, 5 µL. Detection, UV at 225 nm. Peak identification: 1, (±)-octopamine; 2, (±)-synephrine; 3, tyramine; 4, hordenine; 5, *N*-methyltyramine. (From Pellati, F. and Benvenuti, S., *J. Chromatogr. A*, 1165, 58–66, 2007. With permission.)

profiling of the ephedrines present as impurities in, for example, methamphetamine, which is prepared from (1*R*,2*S*)-(–)-ephedrine using the Emde method. Makino et al. proposed a phenyl-β-cyclodextrin-type column for this purpose [32]. The obtained result is presented in Figure 32.3.

Since phenylethylamine alkaloids are psychoactive compounds, a fully validated method for a simple, fast, and reliable analysis is essential. Kikura-Hanajiri et al. proposed methods for analyses of 19 drugs with potentially advertised psychotropic/psychoactive effects; these could be useful for the investigation of the distribution of the noncontrolled psychotropic tryptamines and phenylethylamines on the market. The forms of these products were varied and included tablets, powders, liquids (drinks or colored aromatic liquids), resins, sprays, and dried plants/mushrooms. For a successful chromatographic separation of the 19 compounds (11 tryptamines/β-carbolines and 8 phenylethylamines), a C_{18} column and a gradient elution mode was used [2]. A relatively good separation was obtained in 60 min (Figure 32.4).

Schaneberg et al. adapted HPLC for a chemical fingerprint of botanicals for authentication of the plant materials [8]. The fingerprint method was validated by testing a number of populations within a particular species of *Ephedra*. The created method led to the isolation of two new compounds

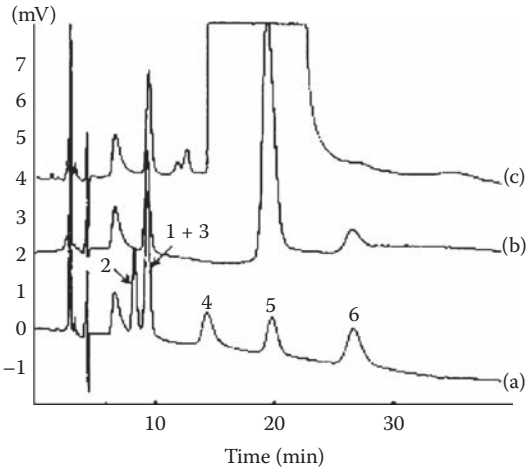


FIGURE 32.3 Chromatograms obtained using the phenyl-β-cyclodextrins-type column. (A) Mixture of standard substances. The peaks correspond to 1 = (1S,2S)-(+)-pseudoephedrine, 2 = (1S,2R)-(+)-ephedrine, 3 = (1R,2S)-(–)-ephedrine, 4 = racemic methylephedrine, 5 = (S)-(+)-methamphetamine, and 6 = (R)-(–)-methamphetamine. (B) Crystal of methamphetamine seized in September 2000 in Tokyo. (C) Crystal of methamphetamine purchased from Dainippon Pharmaceutical. (From Makino, Y., Uranob, Y., and Nagano, T., *J. Chromatogr.* A, 947, 151, 2002. With permission.)

from *E. sinica*. Additionally, this fingerprinting allows distinguishing between *Ephedra* species from Eurasia, North America, and South America.

HPLC with ultraviolet [33,34], photodiode array [35], or MS detection [36–39] has been used to determine colchicine in biological samples. Korner and Kohn [24] elaborated a method of quality control and stability testing of a standardized extract of *Colchicum* seeds and different formulations. The method permits assaying of alkaloids together with their degradation products including colchicine without prior derivatization. Optimization was performed using Drylab software.

Rosso and Zuccaro [34] proposed an effective reversed-phase HPLC method for analysis of an alkaloid mixture containing some thiocolchicine derivatives. Analysis was performed on a Hypersil C_{18} BDS, 5 μm, 100 × 4.6 mm, supplied by CPS, using the gradient elution mode (Figure 32.5). Electrospray positive ionization mass spectrometry in the multiple reaction monitoring (MRM) mode enabled a limit of detection at the level of 35 pg/mL to be achieved for determination of colchicine in human plasma [13]. UV detection at 254 nm allows the obtaining of a limit of quantitation equal to 0.025% (w/w) for demecolcine and colchicine and 0.05% (w/w) for colchicine [24].

Recent technological advances have made available reversed-phase chromatographic stationary phases with a 1.7 μm particle size that can operate at much higher pressures. This technology, termed ultra performance liquid chromatography (UPLC), offers significant advantages in resolution, speed, and sensitivity for analytical determinations, particularly when coupled with mass spectrometers capable of high-speed acquisitions. Churchwell et al. explored these differences by conducting a comparison of UPLC with LC-MS methods, previously optimized for HPLC-based separation and quantification. In general, UPLC produced significant improvements in method sensitivity, speed, and resolution, also in relation to diastereomers, which were not manageable using HPLC [40]. UPLC separation of sympathomimetic *Ephedra* alkaloids was performed by the use of isocratic elution with 2% acetonitrile and 98% 0.1% formic acid (aq.) (total run time 4 min); HPLC separation was achieved on a Luna C_{18}-2 column (2 × 150 mm, 3 μm particles, Phenomenex) eluted at 200 μL/min with an isocratic elution using 4% acetonitrile in 0.1% formic acid (aq.). The entire effluent was directed into the mass spectrometer (total run time 15 min). The isocratic HPLC method had adequate resolution to separate to baseline the diastereomers ephedrine and pseudoephedrine, but this required use of a 2 × 150 mm column and a total run time of 12 min. By comparison, the

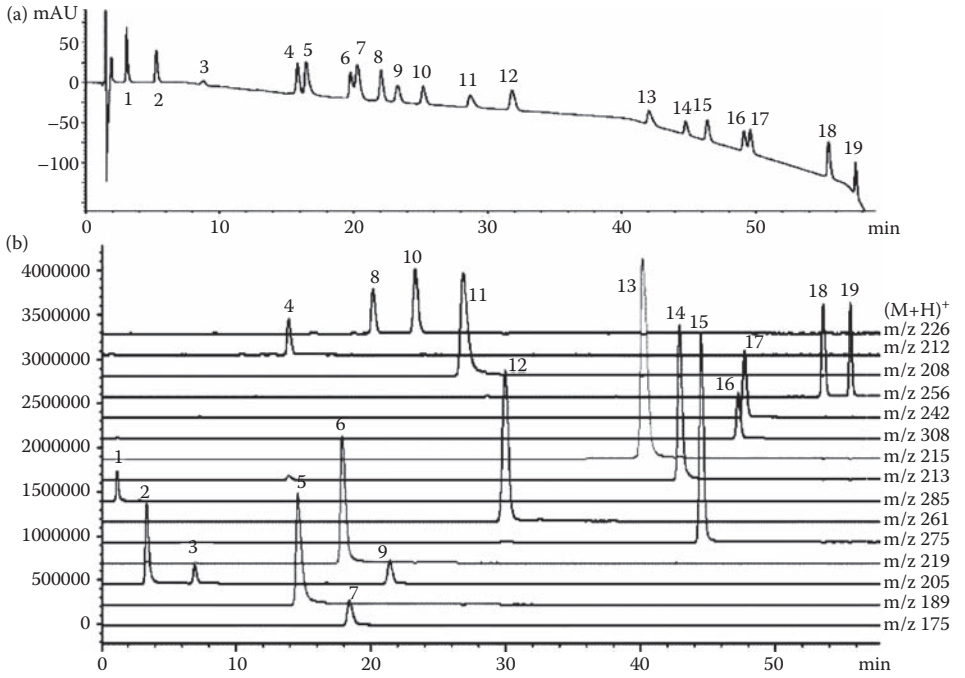


FIGURE 32.4 HPLC-UV chromatograms of a mixed standard solution of 18 drugs (50 μg/mL): (1) Psilocybin; (2) bufotenine; (3) psilocin; (4) mescaline; (5) DMT; (6) 5-MeO-DMT; (7) α-methyltryptamine-AMT; (8) TMA; (9) 5-methoxy-α-methyltryptamine-5-MeO-AMT; (10) TMA-2; (11) MBDB; (12) *N,N*-diisopropyl-4-hydroxytryptamine 4-OH-DIPT; (13) harmaline; (14) harmine; (15) 5-MeO-DIPT; (16) 2C-I; (17) 2C-T-2; (18) 2C-T-4; and (19) 2C-T-7. Stationary phase: Atlantis d C_{18} column. Mobile phase: (A) 10 mM ammonium formate (pH 3.5)–acetonitrile (95:5, v/v) and (B) acetonitrile–methanol (7:3, v/v) delivered at 0.3 mL/min; ratio of A to B: 100:0 (0 min) to 95:5 (15 min) to 90:10 (35 min) to 73:27 (52 min) to 30:70 (60 min). Detection: a tandem setting of photodiode array detector (PDA) and a mass detector (MSD). (DMT, *N,N*-Dimethyltryptamine; 5-MeO-DMT, *N,N*-dimethyl-5-methoxytryptamine 3,4,5-trimethoxyamphetmine TMA; TMA-2, 2,4,5-Trimethoxyamphetmine; MBDB, 2-methylamino-1-(3,4-methylenedioxyphenethyl) butane; 5-MeO-DIPT, *N,N*-Diisopropyl-5-methoxytryptamine; 2C-I, 2, 5-Dimethoxy-4-iodophenethylamine; 2C-T-2, 4-ethylthio-2,5-dimethoxyphenethylamine; 2C-T-4, 2,5-dimethoxy-4-isopropylthiophenethylamine; 2C-T-7, 2,5-dimethoxy-4-n-propylthiophenethylamine.) (From Kikura-Hanajiri, R.,Hayashi, M., Saisho, K., and Goda, Y., *J. Chromatogr. B*, 825, 29, 2005. With permission.)

isocratic UPLC method required a run time of 4 min to achieve a similar degree of peak separation. In addition, sensitivity improvements were observed for all components (individual signal-to-noise ratio (*S/N)* increases are the following: norephedrine (phenylpropanolamine), 1.5-fold; ephedrine, 4.8-fold; pseudoephedrine, 4.7-fold; and methylephedrine, 2.6-fold increase). Comparison of HPLC and UPLC chromatograms obtained for ephedrine enantiomers is presented in Figure 32.6.

32.1.4 Enantioselective Chromatographic Methods

Each ephedrine exists as a pair of enantiomers, which may differ in pharmacological activity as well as in the rate of their metabolism. For example, the stimulant effect of the (1*S*,2*R*)-(+)-ephedrine enantiomer amounts to 80% of the activity of the (1*R*,2*S*)-(–)-ephedrine.

Generally, enantiomer separation is based on either diastereomer formation by reaction with a homochiral reagent, followed by the separation of the diastereomeric derivatives on an achiral stationary phase, or diastereomer formation by use of a chiral mobile-phase additive or a chiral stationary phase. Traditionally, for the analysis of ephedrines at low concentration levels existing

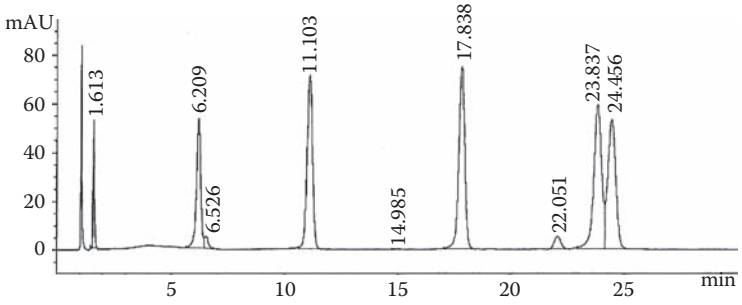


FIGURE 32.5 Analysis of a mixture of: RT 1.61 colchicoside; RT 6.21 thiocolchicoside (TCD); RT 6.53 *N*-deacetyl,*N*-formyl TCD; RT 11.1 colchicine; RT 17.843 *O*-demethyl TCD; RT 22.05 β Lumi-Colchicine; RT 23.84 thiocolchicine (TCN); RT 24.46 *N*-deacetyl, *N*-formyl TCN. The mobile phase was composed of (A) ACN, (B) a solution KH_2PO_4 5 g L^{-1} adjusted to pH 4.5 with phosphoric acid, and (C) THF, delivered according to a linear gradient profile (solvent ratio as v/v): 0–7 min, A:B 0.5:93.5→4:90; 7–20 min, A:B→12:82; 20–26 min, A:B 12:82; 26.1–30 min, A:B→0.5:93.5, with C being held constant at 6%. The flow rate was set at 1 mL/min, the injection volume at 7 μL, the oven temperature at 35ºC, detection at 380 nm. (RT, retention time) (From Rosso, A. and Zuccaro, S., *J. Chromatogr. A*, 825, 96, 1998. With permission.)

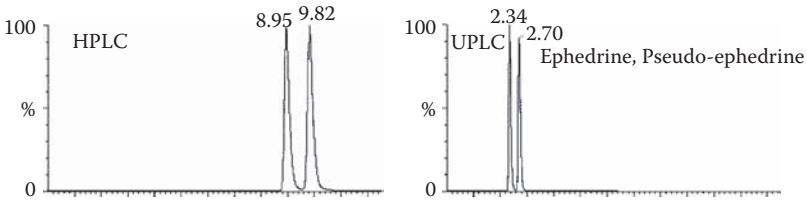


FIGURE 32.6 Comparison of high performance liquid chromatography–tandem mass spectrometry and ultra performance liquid chromatography–tandem mass spectrometry separations of ephedrine and pseudoephedrine. (From Churchwell, M.L., Twaddle, N.C., Meeker, L.R., and Doerge, D.R., *J. Chromatogr. B*, 825, 134, 2005. With permission.)

in biofluids, indirect methods are utilized. Successful results have been obtained with such chiral reagents as 2,3,4,6-tetra-o-acetyl-β-d-glucopyranosyl isothiocyanate,-(+)-1-(fluorenyl) ethylchloroformate, or 9-fluorenylmethylchloroformate-l-proline [12].

Herráez-Hernández and Campíns-Falcó applied β-cyclodextrins (β-CDs)—native β-CD, methyl-β-CD, carboxyethyl-β-CD, carboxymethyl-β-CD, and hydroxypropyl-β-CD—for the direct enantioseparation of some ephedrines: norephedrine, ephedrine, pseudoephedrine, *N*-methylephedrine, and and *N*-methylpseudoephedrine. In this work the employment of an achiral stationary phase with immobilized β-CD and the addition of a chiral selector to the mobile phase have been compared [15].

For the enantioseparation of synephrine in *C. aurantium* fruits, extracts, and natural products, Pellati et al. [41] also proposed a β-CD stationary phase. HPLC analysis was performed on a LiChroCART Chiradex column (250 × 4.0 mm ID, 5 μm); the mobile phase was methanol–25 mM NaH_2PO_4, pH 3.5 (20:80, v/v) and 10 mM tetrabutylammonium hydrogen sulfate in the ratio of 30:70 (v/v). The column was thermostatically controlled at 3°C. Detection was performed at 220 nm. Unfortunately, under these chromatographic conditions, only a partial enantioseparation of synephrine was obtained: α (selectivity factor) was 1.05 and R_s (peak resolution) was 0.73. More recently, Pellati et al. [42] performed a satisfactory separation of synephrine enantiomers using a protein-based chiral stationary phase. Sample preparation was performed by magnetic stirring with water. The extract was cleaned up by SPE on a LiChrolut SCX column. HPLC analyses were carried out on a Chiral-CBH column (100 × 4.0 mm ID, 5 μm), with a mobile phase of 5% (w/w) 2-propanol

in 10 mM sodium phosphate (pH 6.0) and 50 μM disodium EDTA, at a flow rate of 0.8 mL/min. Detection was set at 225 nm. The retention time was 3.37 min for (–)-synephrine and 4.28 min for (+)-synephrine. The α value was 1.27 and R_s was 2.11.

Bielejewska et al. proposed the addition of (+) or (–)-camphorsulfonic acids (CSAs) for the enantioseparation of basic drugs, among others ephedrine enantiomers, on a Chiralcel OD column and a Nucleosil column. The authors examined the effects of small additions of acetic acid (AcOH) or (+) or (–)-CSAs together with water to the mobile phase consisting of hexane and 2-propanol (IPA) (80:20, v/v) [43].

32.1.5 HPLC-MS

The use of chromatographic separation coupled with mass spectrometry for the chemical characterization and composition analysis of botanicals has been growing rapidly in recent years. The use of hyphenated techniques, such as HPLC-MS and HPLC–tandem mass spectrometry (HPLC-MS/MS) to perform on-line composition and structural analyses has provided a wealth of information unsurpassed by other techniques.

He et al. [44] studied an HPLC–electrospray ionization (ESI)–MS technique for the qualitative analysis of flavonoids and synephrine in *C. aurantium* fruits. Samples were extracted with 80% ethanol at 90°C. A Symmetry C_{18} column (150 × 2.1 mm ID, 5 μm) and a gradient mobile-phase of 0.6% acetic acid aqueous solution and methanol were employed. ESI–quadrupole MS detection operating in full-scan mode was applied.

HPLC-MS/MS has been applied for the analysis of ephedrine alkaloids and synephrine in standard reference materials [45] or dietary supplements. Gay and White [26] reported a simplified analytical method based on HPLC–atmospheric pressure chemical ionization (APCI)–MS/MS that uses selected reaction monitoring (SRM) for determination of ephedrine alkaloids and synephrine in dietary supplements. The samples were extracted with 80% methanol by sonication. After sonication, two alternative procedures were applied: The first included a cleanup of the extract by SPE on an Isolute SCX-2 column; the second was based on a simple filtration. For chromatography, a YMC phenyl column (250 × 2.0 mm ID, 5 μm) was used, with a mobile phase consisting of 2% acetic acid, 44 mM ammonium acetate, and 3% acetonitrile (ACN), at a flow rate of 0.23 mL/min. A triple-quadrupole mass spectrometer was used for detection, with interchangeable ESI and APCI sources. Calibration curves were constructed by using an isotopically labeled internal standard (–)-(1*R*,2*S*)-ephedrine.

Beyer et al. [21] validated an HPLC-ESI-MS/MS method to detect and quantify ephedrine alkaloids and synephrine in plasma. After mixed-mode SPE of 1 mL of plasma, the analytes were separated using a Zorbax SCX column (150 × 2.1 mm ID, 5 μm), with a gradient mobile-phase consisting of 5 mM aqueous ammonium formate (pH 3.0) and ACN, at a flow rate of 1.5 mL/min. A hybrid triple-quadrupole/linear ion trap mass spectrometer, operating in the multiple reaction monitoring (MRM) mode, was used for detection. Calibration curves were constructed by using isotopically labeled internal standards (norephedrine, ephedrine, and mescaline).

High-performance liquid chromatography-multistage tandem mass spectrometry (HPLC-MS^n) has been used successfully for the determination of phenylethylamine alkaloids in complex matrices, such as natural products. Mattioli et al. [46] described an HPLC-ESI-MS^n method for the analysis of synephrine in dry extracts of *C. aurantium* fruits and in fresh fruits and juices of *C. sinensis*. *C. aurantium* samples were extracted with the mobile phase at 90°C. *C. sinensis* samples were sonicated with the mobile phase at 30°C. For HPLC analysis, a Prodigy RP-18 column (250 × 4.6 mm ID, 5 μm) was used; the mobile phase was water–ACN–formic acid (64.9:35:0.1), and the flow rate was 0.8 mL/min in isocratic conditions. An ion trap mass selective detector was used for identification.

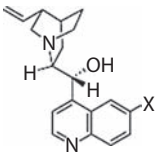
Jiang et al. described a rapid, sensitive, and selective LC-MS/MS method for the determination of colchicine in human plasma. The assay requires only a small sample volume (0.10 mL), achieving a limit of quantitation of 0.050 ng/mL in a short run time of 2.5 min [13].

32.2 QUINOLINE DERIVATIVES

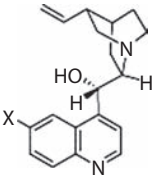
32.2.1 *Cinchona* Alkaloids

The *Cinchona* alkaloids are a group of about 35 bases occurring in the bark of *Cinchona* and *Remijia* species. The principal *Cinchona* alkaloids are the quinoline alkaloids: quinine (14), quinidine (15), cinchonine (16), and cinchonidine (17).

Reversed-phase HPLC, using ODS columns in combination with acidic mobile phases, and UV detection, is the most widely used chromatographic procedure for the qualitative and quantitative analysis of these compounds in a variety of samples. An extensive review devoted to chromatographic determination of *Cinchona* alkaloids was prepared by McCalley [47].



(14) Quinine X = OCH_3



(15) Quinidine X = OCH_3

(16) Cinchonidine X = H

(17) Cinchonine X = H

32.2.1.1 Extraction Procedures

The extraction of the major alkaloids from *Cinchona* bark is usually performed after preliminary pulverizing, grinding, sieving, and drying of the bark at 110°C followed by treatment with alkali (lime, calcium hydroxide, sodium or ammonium hydroxide) and Soxhlet extraction in hot toluene [48], benzene, or methanol [49]. This method was modified by Haznagy, who claimed that trichloroacetic acid in methanol should be preliminary used, followed by treatment with a base and organic solvent extraction [50]. In biomedical studies, quinine and quinidine are extracted from plasma or urine also by making the sample basic with, for instance, sodium or ammonium hydroxide and extracting into an organic solvent such as dichloromethane or diethyl ether [51,52].

For cell suspension cultures containing low levels of alkaloids, the following method was proposed: Cell samples were homogenized in 0.2 *M* sulfuric acid and washed with chloroform to remove impurities while the alkaloids were presumed to remain in the acid aqueous phase. The aqueous extract is made basic (e.g., with ammonium hydroxide) and again extracted with an organic solvent [53].

32.2.1.2 Separation Methods

McCalley [47,54] presented a comparison of different reversed-phase columns including conventional C_{18} phases, phases with embedded polar groups, short-chain and cyano phases, and a high-pH-stable phase according to such parameters as retention factor, column efficiency, and asymmetry factor for, among others, quinine using acetonitrile–0.0265 *M* phosphate buffer, pH 3.0 (15:85, v/v). Considerable variation was observed in the peak shape and column efficiency, ranging from extreme peak asymmetry ($A_s > 5.0$) and very poor efficiency (500 plates) for Hypersil ODS to good peak asymmetry ($A_s < 1.4$) and high-column efficiency (15,000–20,000 plates) for the most recently introduced "new-generation" phases. Of the "classical phases" only μ-Bondapak C_{18} gave a reasonable peak shape. Generally, for quinine, shorter-alkyl-chain C_8 phases have been found to be more advantageous than C_{18} phases. LiChrosorb RP 8 Select B can be seen to give improved results for quinine as compared with other investigated phases [55]. A separation obtained on this phase is presented in Figure 32.7.

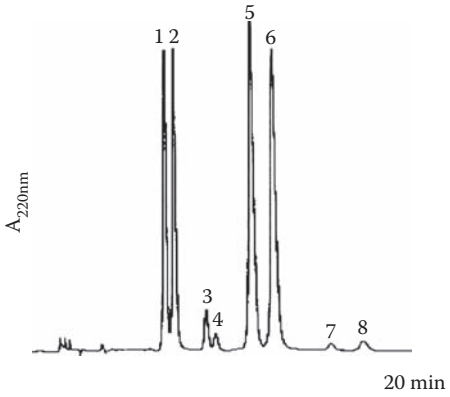


FIGURE 32.7 HPLC separation of alkaloids on the LiChrosorb RP-8 Select B column. Peaks: 1 = cinchonine, 2 = cinchonidine, 3 = dihydrocinchonine, 4 = dihydrocinchonidine, 5 = quinidine, 6 = quinine, 7 = diydroquinidine, 8 = dihydroquinine. Detection, UV at 220 nm. Flow rate, 1 mL min^{-1}. Eluent, 15% acetonitrile in 0.1 *M* phosphate buffer adjusted to pH 3.0 with phosphoric acid before modifier addition (From McCalley, D.V., *J. Chromatogr.*, 357, 221, 1986. With permission.)

According to McCalley, the worst results such as tailing peaks, poor column efficiency, or irreproducible retention times are a consequence of silanophilic interactions between the underivatized column silanol groups and protonated alkaloid molecules. These disadvantageous effects could be improved by the use of mobile-phase additives or phases of low silanol activity, for instance, shorter alkyl chains or cyanopropyl columns. To eliminate interaction with free silanols, some researchers have used a mobile phase with a low pH (2–3), enabling suppression of silanol groups' ionization. However, on a modern RP column, it is possible to work in a wide range of pH environments. Successful analysis of the *Cinchona* alkaloids can even be obtained on classical reversed-phase HPLC silica by addition of amines like triethylamine to the mobile phase; these compete with the analytes for column silanol sites [56]. For example, Hypersil ODS was used to give a complete separation of the four major *Cinchona* alkaloids and their corresponding dihydro derivatives using a pH 3 buffer containing 0.05 *M* hexylamine and acetonitrile [57]. The same classical phase was used successfully for the analysis of quinine and its major metabolite, 3-hydroxyquinine, in human plasma and urine using an acetonitrile–phosphate buffer, pH 2.1, containing tetrabutylammonium bromide [58]. Gatti et al. [59] proposed using the Prodigy column presenting low silanol activity for analysis of cinchona alkaloids. The ultra-low-metal-content silica (99.999% pure) and an inert bonded surface eliminate the need for mobile-phase modifiers or ion-pairing reagents. Reversed-phase separation of the *Cinchona* alkaloids cinchoninone, quinidinone, cinchonine, cinchonidine, quinine, and quinidine can also be accomplished by the use of Li-Chrosorb RP-8 Select B and a mobile-phase containing 1*M* sodium acetate buffer–acetonitrile–tetrahydrofuran (80:20:5, pH 5.5–6.0) [60]. Table 32.3 covers some selected HPLC methods for determination of *Cinchona* alkaloids.

32.2.1.3 Detection of Alkaloids in HPLC Analysis

The majority of authors have used UV detection, which can be considerably improved by the use of photodiode array detectors, generating complete analyte spectra. Difficulties in detection of *Cinchona* alkaloids are connected with the change in the spectra occurring with pH for these ionogenic compounds. Furthermore, the spectra of the pairs quinine/quinidine and cinchonine/cinchonidine are identical.

Biomedical studies involving analysis of low amounts of alkaloids and their metabolites in body fluids require much higher detection sensitivity. In these cases, fluorescence detection is much

TABLE 32.3
HPLC Methods for *Cinchona* Alkaloids Determination

Compounds	Extraction Procedure	Recovery	Column/Eluent/Detection	Ref.
Cinchona alkaloids and their dihydro derivatives together with pyridoxine hydrochloride (vitamin B_6)	1. An amount of 20 mg of the powder was treated with 40 mL of methanol and 0.5 mL of sodium hydroxide by ultrasonication for 20 min at ambient temperature and diluted to 50 mL with methanol. The resulting suspension was filtered; 1 mL of the clear solution was diluted to 10 mL with mobile phase for HPLC analyses. 2. An amount of 20 mg of powder was weighed and transferred into a Soxhlet using 40 mL of methanol and 0.5 mL of sodium hydroxide. Samples were Soxhlet extracted for 7 h, and the extract was diluted to 50 mL with methanol; then 1 mL of the resulting solution was diluted to 10 mL with mobile phase and then injected into the chromatograph.	97.8%–105%	Phenomenex Prodigy ODS column, 5 µm, 250 × 3.2 mm Triethanolamine (TEA) phosphate buffer (pH 3.0; 0.05 M)–acetonitrile (88:12, v/v) Fluorescence detection (excitation 330 nm, emission 420 nm)	[59]
Quinine and chloroquine in pharmaceuticals and biological fluids	*Pharmaceutical preparation*: Ten tablets were weighed and finely pulverized. A portion of this powder was transferred to a 50 mL volumetric flask and diluted to volume with water. After sonication, this sample was filtered, and dilute solutions were prepared at three concentrations: 0.5–1.0 and 2.0 ng/µL for quinine and 0.05–0.1 and 0.3 ng/µL for chloroquine. Aliquots of 50 µL were injected onto the chromatographic column. *Solid-phase extraction (SPE) of biological fluids*: 2 mL of acetonitrile is added to 40 µL of sample. 2 mL of water (for blank samples) or of standard (for spiked; at five concentration levels: 0.2, 0.5, 0.75, 1, and 2 ng/µL) were added. After centrifugation for 10 min, organic solvent was evaporated under nitrogen stream, and the remaining sample was applied to the preconditioned cartridge. Same procedure was followed for urine samples. Urine samples of 100 µL were spiked with 100 µL of standard solution at four concentration levels 0.1, 0.2, 0.3, and 0.5 ng/µL. These samples were treated by SPE. Four SPE cartridges: Lichrolut RP-select B, LC-18 Discovery, C_8 Bond Elut, and Nexus Abselut were tested. Cartridges were preconditioned with methanol. For the second conditioning step, water, 0.1 mol/L ammonium acetate, and acetic acid 1%, for elution efficiency: methanol–acetonitrile (50%–50%, v/v), methanol, acetonitrile, acetic acid 1%, methanol–0.1 mL/L ammonium acetate–acetonitrile (45:40:15, v/v), 2-propanol, methanol–acetone (80:20, v/v), 2-propanol–acetonitrile (50:50, v/v), water, hydrochloric acid 1%, and for sequential elution by 1.3 mL of methanol–0.1 mL/L ammonium acetate–acetonitrile (45:40:15, v/v) and 0.7 mL acetic acid 1% were tested.	92.1% for quinine, 105.4% for chloroquine from blood serum 101.8% for quinine, 90.7% for chloroquine from urine	Kromasil C_{18}, µm, 250 × 4 mm Methanol–acetonitrile–0.1 mol/L ammonium acetate (45:15:40, v/v) Fluorescence detection (excitation 325 nm, emission 375 nm)	[61]

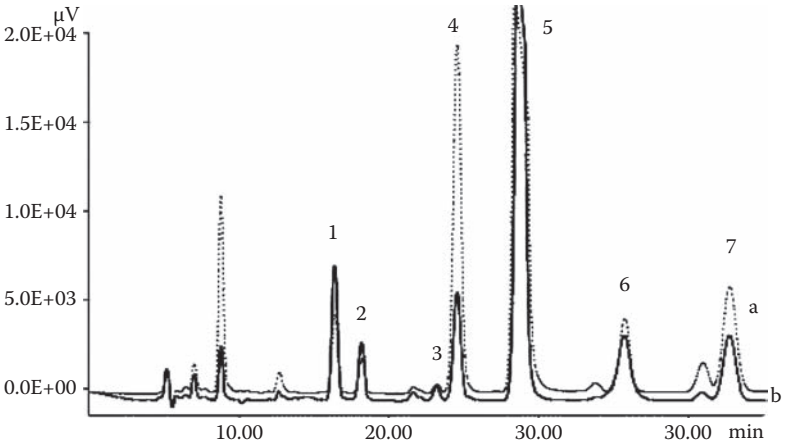


FIGURE 32.8 LC chromatogram of a sample of *Cinchona* bark with on-line photoreactor switched (a) off and (b) on. Peaks: 1, cinchonine; 2, cinchonidine; 3, dihydrocinchonine; 4, quinidine; 5, quinine; 6, dihydroquinidine; 7, dihydroquinine. LC conditions: Phenomenex Prodigy 5 m ODS column (250 × 3.2 mm ID) using triethylamine (TEA) phosphate buffer (pH 3.0; 0.05 M)–acetonitrile (88:12, v/v) as mobile phase; flow rate 0.4 mL min^{-1}. Fluorescence detection: λ_{ex} = 330 nm; λ_{em} = 420 nm. (From Gatti, R., Gioia, M.G., and Cavrini, V., *Anal. Chim. Acta*, 512, 85, 2004. With permission.)

more suitable. Owing to the photochemical conversions, discrimination of quinine-type alkaloids from cinchonine-type alkaloids is possible, as after photoconversion cinchonine-type alkaloid peaks increase, while the peaks of quinine-type alkaloids decrease. A representative separation of *Cinchona* alkaloids with and without UV irradiation is illustrated in Figure 32.8 [59].

MS is the method of choice for the definitive identification of alkaloids, especially when examined at low levels. Giroud et al. [62] used a thermospray MS interface as well as conventional UV detection at 280 nm to investigate alkaloid production in *Cinchona* shoot and compact globular structure cultures. Authors noticed that all the alkaloids gave intense quasimolecular ions (M-H) + but little fragmentation. This is disadvantageous for structural elucidation. Furthermore, some compounds gave acetonitrile adducts $(M\text{-}H\text{-}ACN)^+$.

32.2.1.4 Cinchona Alkaloids as Chiral Selectors

The *Cinchona* alkaloids quinine and quinidine have been used successfully for enantiomer separation either as a chiral ion-pairing additive to the mobile phase or as a chiral stationary-phase ligand [63,64]. Recently, chiral selectors based on carbamoylated derivatives of quinine and quinidine have been found to be stereoselective for the resolution of acidic enantiomers by the use of mobile phases consisting of aqueous buffers and methanol or acetonitrile as organic solvents [65–71]. The structure of this chiral selector is presented in Figure 32.9. These chiral selectors are weak anion exchangers, owing to the presence of a tertiary amino group within the quinuclidine ring. At the acidic pH of the mobile phase these amino groups are protonated, whereas solutes exist in anionic form. A chiral recognition mechanism based on the primary ionic interaction between them is significantly accompanied by additional intermolecular interactions such as hydrogen-bonding, dipole–dipole, charge-transfer, hydrophobic, and steric ones. The authors corroborated their explanation by spectroscopic investigations (Fourier transform infrared (FT-IR) and X-ray). Dimeric versions of chiral stationary phase (CSP), in which two quinine units are connected by a difunctional spacer, have also been prepared [68]. A *tert*-butylcarbamoylated quinine linked to mercaptopropyl silica can separate the enantiomers of *N*-3,5-dinitrobenzoylated leucine and phenylalanine in a buffered aqueous mobile phase with α-values of 15.87 and 10.78, respectively [67,72].

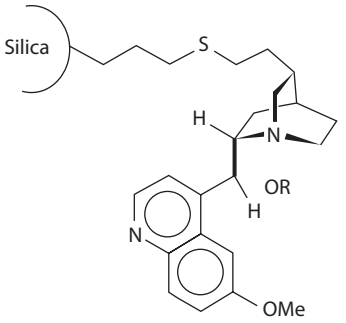


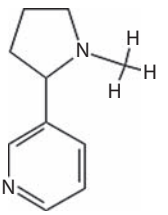
FIGURE 32.9 Chiral stationary phase with *tert*-butylcarbamoylated quinine selector linked to 3-mercaptopropyl silica gel. (From Gasparrini, F., Misiti, D., and Villani C., *J. Chromatogr. A*, 906, 35, 2001. With permission.)

32.3 PYRIDINE AND PIPERIDINE ALKALOIDS

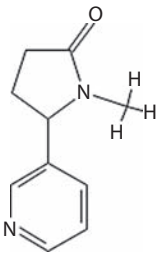
32.3.1 Tobacco Alkaloids

Nicotine (18) is a naturally occurring alkaloid found in the solanaceous family. Nicotine is a diamine composed of pyridine and pyrrolidine rings (pK_a: 7.84, 3.04), occurring also in the form of stereoisomers. The metabolism of nicotine in living organisms is very complex, involving, in the first phase, microsomal oxidation and, in the second phase, *N*- and *O*-glucuronidation of nicotine and its metabolites. Nicotine can be metabolized to more than 20 different derivatives. Cotinine (19) is a major oxidized metabolite identified in urine of man, rabbit, mouse, and rat [73].

Owing to its low boiling temperature, nicotine in tobacco and tobacco products is analyzed by gas chromatography rather than by the liquid chromatographic techniques of thin-layer chromatography (TLC) or HPLC. However, analyses of biological samples containing nicotine metabolites have been performed using liquid chromatography.



(18) Nicotine



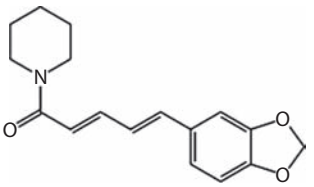
(19) Cotinine

Okoli et al. [74] assessed the validity of hair nicotine as a biomarker for secondhand smoke exposure. Hair samples were analyzed by reversed-phase HPLC with electrochemical detection (HPLC-ECD). Wiergowski et al. [75] elaborated on the procedure for the determination of nicotine and cotinine both in classic (serum, urine) and alternative biological materials (hair, saliva). The liquid–liquid extraction procedure and HPLC-UV-diode array detector (DAD) were applied. Hoofnagle et al. [76] described the sensitive and specific determination of nicotine, its metabolites, and the tobacco alkaloid anabasine in urine. Rapid sample preparation and further HPLC separation of tobacco alkaloids and metabolites were interfaced with tandem mass spectrometry. Moyer et al. [77] also applied this method for quantification of nicotine, cotinine, trans-3'-hydroxycotinine, nornicotine, and anabasine in either serum or urine. HPLC-MS/MS enabled researchers to obtain imprecision < 10% at physiologic concentrations and limits of quantification ranging from 0.5 to

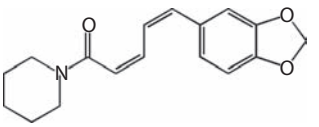
5 µg/L. Mahoney and Al-Delaimy [78] developed an assay for nicotine in hair based on reversed-phase HPLC with electrochemical detection. Analytical parameters were satisfactory, with linearity between 4 and 640 ng mg–1 and recoveries of 105 ± 6.5%; the limit of detection was lower than 0.05 ng. The method uses a low-metal, high-purity silica reversed-phase column (a Prodigy 5 µm ODS, 150 × 4.6 mm). The mobile phase was 50 mmol KH2PO4 with 2.5% methanol and 4% acetonitrile added by volume. The final mixture was adjusted to pH 4.8. Nicotine, cotinine, trans-3'-hydroxycotinine, trans-3'-hydroxycotinine glucuronide, nicotine-1'-N-oxide, and 3-pyridylcarbinol were quantified in urine using norephedrine as an internal standard and UV detection at 260 nm [79]. Urine of pregnant women was also investigated for its tobacco alkaloid contents by HPLC [80]. Table 32.4 presents different procedures for determination of tobacco alkaloids in samples of different origin.

32.3.2 Pepper Alkaloids

Pepper plants accummulate pungent bioactive alkaloids called piperamides. Black pepper contains 5%–9% of an active alkaloid, piperine (20). It undergoes light-induced isomerizations producing the following four possible piperine-derived photo-induced isomers: piperine, isopiperine, chavicine (21), and isochavicine. To determine the kinetics of these isomerizations, HPLC and LC-DAD-MS were applied [89–97].



(20) Piperine



(21) Chavicine

Piperine is a highly lipophilic molecule showing a wide spectrum of pharmacological actions such as antidiarrheal, hepatoprotective, and antioxidant activity. Recently, piperine has been reported to increase the bioavailability of various drugs such as propranolol, theophylline, aflatoxin-B_1, and curcumin. Piperine has also been added to multidrug anti-tuberculosis therapy, where the dose of rifampicin can been reduced by 55% (unpublished data) [89]. Bioavailability enhancement by piperine is due to its potent inhibitory action on drug-metabolizing enzymes. Bhat and Chandrasekhara [90] describe its metabolism to piperic acid by amidase, which is then metabolized to piperonal, piperonylic acid, and vanillic acid. In recent studies, new metabolites were characterized in rat urine using LC-MS/MS [91] and LC–nuclear magnetic resonance (NMR)–MS [89]. The metabolite was partially purified using reversed-phase column chromatography on Sephadex-LH 20 and characterized as 5-(3,4-methylenedioxy phenyl)-2*E*,4*E*-pentadienoic acid-*N*-(3-yl propionic acid)-amide with the help of LC–NMR–positive ESI–MS studies [89]. The HPLC fingerprint profile of piperine metabolites is presented in Figure 32.10. HPLC systems applied for determination of pepper alkaloids are collected in Table 32.5.

32.4 IMIDAZOLE ALKALOIDS

32.4.1 Pilocarpine Type

Several plants of the genus *Pilocarpus* contain the toxic alkaloids pilocarpine, isopilocarpine, pilocarpidine, jaborine, and jaboridine, which possess an imidazole skeleton. The main species is *microphyllus*, and some other minor representatives of the genus are *jaborandi*, *pennatifolius*, *trachylophus*, and *racemosus*. Pilocarpine is a parasympathomimetic compound. It is mainly used in ophthalmic solutions for the treatment of chronic open-angle glaucoma. Pilocarpine stimulates the secretion of saliva, which is why it is also used to treat xerostomia, a side effect of radiation therapy.

TABLE 32.4
Procedures of Determination of Tobacco Alkaloids in Samples of Different Origin

Alkaloid Determined in Different Samples	Extraction Procedure	Recovery/LOD	HPLC System (Stationary Phase/ Mobile Phase/Detection)	Ref.
Nicotine and cotinine in urine	A 10 mL sample of a smoker's urine (20 mL) was mixed with 100 mL of a 0.01 mg/mL 2-phenylimidazole solution in methanol and 0.5 mL of 0.5 M sodium hydroxide. The mixture was shaken and immediately transferred to an Extrelut-20 column. After a minimum of 30 min, the analytes were eluted with 40 mL of chloroform in conical vial containing 100 mL of glacial acetic acid. The acid organic phase was evaporated to dryness at 40ºC under a nitrogen stream, and the residue was reconstituted in 100 mL of mobile phase.	Cotinine, 92%–100%; nicotine, 47%–86% Cotinine: 0.5 ng/mL; nicotine: 5.0 ng/mL	Column: μBondapack C_{18} column Inertsil ODS-3 (250 × 4.6 mm, particle size 5 μm) Mobile phase: acetonitrile–methanol–pH buffer (90:30:880, v/v) containing 0.3 mM of sodium octane sulfonate as ion pair. The pH of the mobile phase was adjusted to 4.8 with triethylamine. Detector monitored at 260 nm	[81]
Nicotine, cotinine, and caffeine in meconium	A meconium aliquot, 2.0 g, was weighed, mixed with ephedrine (used as an internal standard), and emulsified in 20 mL of 0.1 mol/L phosphate buffer (pH 8.0). The suspension was agitated by vortex mixing and then centrifuged. The supernatant was recovered and filtered. The alkaloids being studied were extracted from supernatant using chloroform (332 mL). Chloroform extracts were evaporated to dryness, and the residue was dissolved in 10 mL boric buffer (pH 9.0). The prepared sample was then passed through an additional solid-phase extraction as follows: A C_8 column (3mL) was pretreated by passing methanol (5mL) followed by water (5mL) through it. The C_8 column was then placed onto a silica gel column (3mL) which had been pretreated with chloroform (5mL).The sample was passed through C_8 and then the silica gel column. The column was removed, and the alkaloids were eluted from the silica gel column with 3 mL of 70% methylene chloride plus 30% methanol, containing 1% ammonium hydroxide. After solid-phase extraction, the organic phase was evaporated to dryness under nitrogen and redissolved in 100 mL of water, and 20 mL was injected into the HPLC column.	Cotinine, 85%; nicotine, 90%; caffeine, 92% 10 ng/mL	A steel column (250 × 34.6 mm ID) packed with Lichrosorb C_{18} (particle size 5 μm) Mobile phase: water–methanol—buffer acetate (pH 4.66)–acetonitrile–acetic acid (50:29:20:2:1, v/v), adjusted to pH 4.3 with diethylamine	[82]

(*Continued*)

TABLE 32.4 (CONTINUED)
Procedures of Determination of Tobacco Alkaloids in Samples of Different Origin

Alkaloid Determined in Different Samples	Extraction Procedure	Recovery/LOD	HPLC System (Stationary Phase/ Mobile Phase/Detection)	Ref.
Cotinine and trans-3'-hydroxycotinine in human serum	A solid-phase extraction procedure; internal standard, *N*-ethylnorcotinine	5 ng/mL	A 30-cm reversed-phase column and a mobile phase of water–methanol–0.1 M sodium acetate–acetonitrile (67:24.5:6.5:2, v/v), pH 4.3	[83]
Nicotine and cotinine in urine	The analytes are extracted and purified from the complex and impure matrix in two stages: first, by liquid–liquid extraction and then by solid-phase extraction (C_2 column). Two internal standards (2-phenylimidazole and *N*-ethylnorcotinine) were used.	Nicotine, 85%; cotinine, 87%; *N*-ethylnorcotinine, 87% less than 1 μL^{-1}	"DB" C_8 5-microns-particle column (25 × 0.46 cm) and a mobile phase of phosphate-citrate buffer and acetonitrile (91:9) containing 5 mL of triethylamine and 600 mg of heptanesulfonate per liter, adjusted to pH 4.4, to separate the compounds. UV detection (256 nm)	[84]
Nicotine and cotinine in human hair	Basic and acid digestion of hair followed by solid-phase extraction with an Extrelut-3 glass column using dichloromethane–isopropyl alcohol (9:1) as eluting mixture. Addition of methanolic HCl to the eluting mixture prevented the loss of nicotine during the evaporation step before chromatography.	—	Chromatography was performed using a reversed-phase column and UV detection at 254 nm.	[85]
Nicotine, cotinine, 3-hydroxycotinine, and caffeine from urine samples	Sample pretreatment followed standard protocols, including addition of base before liquid–liquid partitioning against dichloromethane on a solid matrix, evaporation of the organic solvent using gaseous nitrogen, and transferring to HPLC vials using HPLC buffer.	Cotinine, 99%; 3-hydroxycotinine, 78% 1 μL^{-1} except for nicotine (10 μL^{-1} of urine)	HPLC separation was run on-line with electrospray ionization–tandem mass spectrometric detection.	[86]

Nicotine, cotinine in rat blood and brain tissue	Microdialysis probes were inserted into the jugular vein, right atrium, and brain striatum of Sprague–Dawley rats, and nicotine (2 mg/kg, i.v.) was administered via the femoral vein. Dialysates were collected every 10 min and injected directly into a HPLC system.	LOQ (nicotine and cotinine) were 0.25 µg/mL and 0.05 µg/mL	Reversed-phase Luna phenyl-hexyl column (150 × 4.6 mm). Mobile phase: acetonitrile–methanol–20 mM monosodium phosphate buffer (55:45:900, v/v/v, pH adjusted to 5.1 by orthophosphoric acid), 0.5% 1 M citric acid, 0.1% diethylamine, 1 mM octanesulfonic acid. UV wavelength was set at 260 nm.	[87]
Nicotine, cotinine, *trans*-3′-hydroxycotinine, their corresponding glucuronide conjugates, and cotinine-*N*-oxide, norcotinine, and nicotine-*N*′-oxide in the urine of smokers	Urine samples were thawed overnight at 4°C and thoroughly mixed. Aliquots were transferred into plastic tubes, and the internal standards were added to yield a final concentration of 1000 ng/mL. The tubes were mixed for 2 min. The samples were centrifuged for 15 min. The supernatants were filtered. The filtrates were transferred into amber glass vials. Aliquots of 1 µL were injected into the LC-MS/MS system.	Calibration range of 10–5000 ng/mL Recovery: 87%–113% LOD: 0.06 nmol/mL	LC-MS/MS applying atmospheric-pressure chemical ionization (APCI) in the positive-ion mode. Synergy MAX RP (dodecylsilyl modified silica, endcapped, 80 Å pore size, 4 µm particle size, 150 × 4.6 mm ID) analytical column with a guard column (MAX RP, 4 µm particle size, 4 × 3 mm) operated at 45°C oven temperature. Mobile phase: 10 m *M* aqueous ammonium acetate (pH 6.8)–methanol (20:80, v/v).	[88]

Note: LOD, limit of detection; LOQ, limit of quantitation.

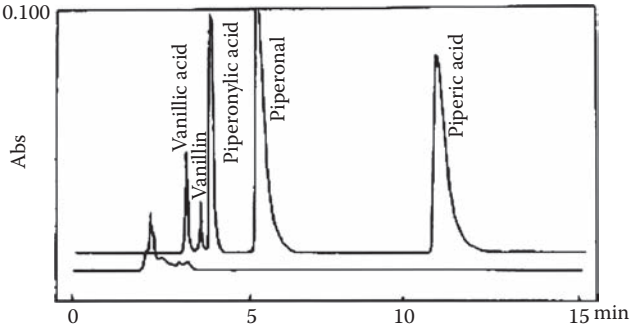


FIGURE 32.10 HPLC fingerprint profile of reported piperine metabolites in rats. Lower chromatogram shows control urine sample. Metabolites were separated using Nova-Pak CN HP column (3.9 × 300 mm, 5 mm) with a solvent system of water–methanol–acetic acid (95:5:0.1, v/v) at a flow rate of 1 mL/min. Samples were monitored at 300 nm. (From Bajad, S., Coumar, M., Khajuria, R., Suri, O., and Bedi, K.L., *Eur. J. Pharm. Sci.*, 19, 413, 2003. With permission.)

In aqueous solution pilocarpine degrades by epimerization to isopilocarpine. The hydrolysis of these two compounds yields pilocarpic and isopilocarpic acids (Figure 32.11). Competition between the degradation routes is dependent on both pH and temperature. It should be stressed however that degradation products of pilocarpine are pharmacologically inactive [99].

The HPLC method can be used for the determination of either pilocarpine or its degradation products in samples of different origin: pharmaceutical preparations, biofluids [99–101], and botanicals. Typically, the following columns are employed: octadecyl [102,103], phenyl [104,105], cyano [106] unmodified silica [107], or β-cyclodextrin [108] in combination with an acidic (pH 2.5–4.0) eluent. Fan et al. [104] tested several C_{18} columns and revealed that only YMC Pack ODS-Am and Supelco LC-18-DB were capable of achieving baseline resolution of pilocarpine and its degradation products. In turn, ElDeeb et al. [109] compared the conventional C_{18} column Superspher RP-18e with a monolithic one—Chromolith Performance RP-18e—with respect to the resolution of pilocarpine degradation products. Lower limits of detection and quantitation were obtained by the monolithic columns (Figure 32.12). This phenomenon was explained as due to the lower background noise obtained with the second columns, probably because of the better skeleton rigidity of the monolithic network.

The lack of chromophoric groups requires low detection wavelengths, about 215 nm. Recently, precolumn derivatization with 4-bromomethyl-7-methoxycoumarin and further separation of compounds on a cyanopropyl silica column with fluorescence detection has been described. The limit of detection was 1 ng/mL with linearity up to a concentration of 150 ng/mL. Pilosine was used as an internal standard in this method [101]. Van de Merbel et al. described a method for the simultaneous determination of pilocarpine, isopilocarpine, pilocarpic acid, and isopilocarpic acid at the relevant levels in human plasma and urine. The method is based on a simple sample-preparation step—ultrafiltration for plasma and dilution for urine samples—followed by a reversed-phase LC separation of the analytes and detection by means of tandem mass spectrometry (MS/MS), using atmospheric pressure chemical ionization (APCI). HPLC using a 5 μm Inertsil ODS column and a mixture of a 0.05 M ammonium acetate buffer (adjusted to pH 4.0 with trifluoroacetic acid) and acetonitrile (97:3, v/v) was applied in this method [99].

32.5 ALKALOIDS DERIVED FROM MISCELLANEOUS HETEROCYCLIC SYSTEMS

32.5.1 Xanthine Alkaloids

The methylxanthines caffeine (1,3,7-trimethylxanthine) (24), theobromine (3,7-dimethylxanthine) (23), and theophylline (1,3-dimethylxanthine) (22) are the main alkaloids naturally presents in

TABLE 32.5
Determination of Pepper Alkaloids by HPLC

Sample Source	Compounds	Methodology	Results	Ref.
Ground black peppers	Four possible piperine-derived photo-induced isomers: piperine, isopiperine, chavicine, and isochavicine	HPLC and liquid chromatography–mass spectrometry	The limits of detection (LOD) were approximately 15–30 ng for each isomer. Recoveries of spiked samples were as follows: chavicine, 98.4 ± 2.1%; isopiperine, 96.2 ± 3.2%; piperine, 104 ± 3.8%; isochavicine, 98.9 ± 3.0%	[93]
Piper nigrum L., and in wild species from Central America	Pellitorine, piperylin, 4,5-dihydropiperlonguminine, piperlonguminine, 4,5-dihydropiperine, piperine, and pipercide	Reflux extraction provided the highest recovery of piperine (> 80%) from leaf and peppercorn material. HPLC analysis using a binary gradient of acetonitrile and water separated the major amide peaks between 5 and 12 min. Atmospheric pressure chemical ionization (APCI)-MS improved the detection limit to 0.2 ng, 10-fold below the 2 ng limit of the HPLC-diode-array detector (DAD) based on linear standard curves between 0.1 and 250 µg/mL ($R^2 = 0.999$).	Atmospheric pressure chemical ionization (APCI)-MS improved the detection limit to 0.2 ng, 10-fold below the 2 ng limit of the HPLC-diode array detector (DAD) based on linear standard curves between 0.1 and 250 µg/mL ($R^2 = 0.999$).	[94]
Piper longum L.	(2*E*,4*E*)-*N*-isobutyl-eicosa-2,4-dienamide, (2*E*,4*E*,14*Z*)-*N*-isobutyl-eicosa-2,4,14-trienamide, (2*E*,4*E*,12*Z*)-*N*-isobutyl-ocatadeca-2,4,12-trienamide, guineensine, pipernonaline, pellitorine piperine, piperanine, piperlonguminine	After the saponification by KOH of the ethanol extracts solution of 15 kg of crude drug "Piper Longi Fructus," the fruits of *P. longum* L., the solution was extracted with light petroleum and 500 g of red crude oil was obtained. Using 2.5 g of red crude oil as a sample, the preparative upright countercurrent chromatography (CCC) with a two-phase system composed of light petroleum (bp 60–90°C)–ethyl acetate–tetrachloromethane–methanol–water (1:1:8:6:1, v/v) was successfully performed, which yielded nine fractions. Then these fractions were further purified by use of reversed-phase liquid chromatography (RPLC) with a glass column of 500 × 10 mm ID packed with reversed-phase silica gel. As a result, nine target amides with over 95% purity were obtained.	Countercurrent chromatography and reversed-phase liquid chromatography	[95]

(*Continued*)

TABLE 32.5 (CONTINUED)
Determination of Pepper Alkaloids by HPLC

Sample Source	Compounds	Methodology	Results	Ref.
Egg yolk and albumen	Piperine and its isomers	The absolute detection limit of a standard solution of piperine was 370 pg piperine. The correlation coefficients for the linear calibration graphs (concentration range: $c = 100$ ng–10 µg piperine isomer/mL) are generally better than 0.996. The detection limit for piperine (24.8±0.2 ng/g egg yolk and 37.9±4.9 ng/g albumen) and the recoveries of piperine (70.3±7.7% egg yolk and 75.7±1.9% albumen) were determined.	HPLC and detection using UV, diode-array detection (DAD), and electrochemical detection. Piperine isomers were characterized and identified by spectroscopy (MS, ^{1}H-nuclear magnetic resonance (NMR), Fourier transform infrared (FT-IR)	[96]
Rat urine and plasma	5-(3,4-Methylenedioxy phenyl)-2*E*,4*E*-pentadienoic acid-*N*-(3-yl propionic acid)-amide	The metabolite was partially purified using reversed-phase column chromatography on Sephadex ((R))-LH 20	Liquid chromatography–nuclear magnetic resonance (NMR)–positive-ion electrospray ionization (ESI)–mass spectrometry	[89]
Rat plasma	Piperine	4 mL ethyl acetate and extraction time of 3 min. Analysis was performed using a Symmetry C_{18} column (250 × 4.6 mm) by isocratic elution with 25 mM KH_2PO_4 (pH 4.5)–acetonitrile (35:65) and UV detection at 340 nm.	The calibration plot was linear over the range studied (2–2000 ng) with correlation coefficient of 0.9984. Limits of detection and of quantitation were 1 ng/mL and 3 ng/mL, respectively. Good overall recovery (85.5±6%) was obtained.	[97]
Rat plasma and hepatocyte culture	Ketoconazole and piperine	Analysis was performed using a Symmetry C_{18} column (150 × 4.6 mm, 5 µm) and isocratic elution with 25 mM KH_2PO_4 (pH 4.5)–acetonitrile (50:50) with a flow rate of 1 mL/min. Photodiode-array detection was used to simultaneously monitor piperine at 340 nm and ketoconazole at 231 nm in a single sample.	Calibration plots in spiked plasma, hepatocytes and William's medium E were linear over the range studied (10–2000 ng for both drugs). The detection limits for piperine and ketoconazole are 2 and 4 ng, respectively, and the limits of quantitation are 10 and 12 ng, respectively.	[98]

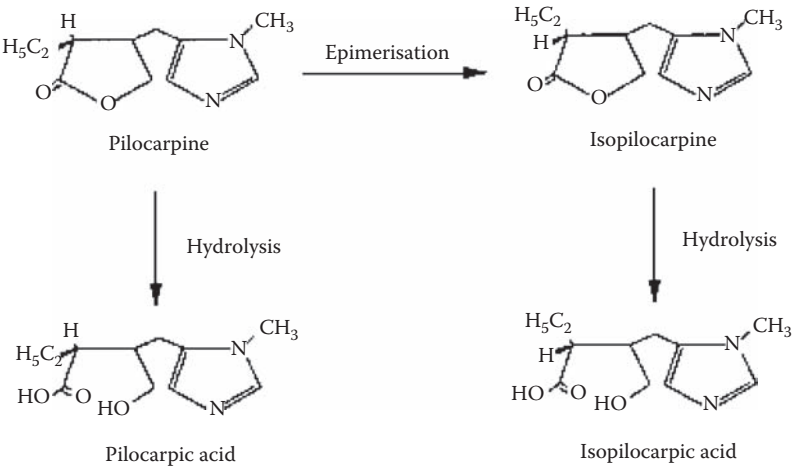


FIGURE 32.11 Degradation pathways of pilocarpine. (From van de Merbel, N.C., Tinke, A.P., Oosterhuis, B., and Jonkman, J.H.G., *J. Chromatogr. B*, 708, 103, 1998. With permission.).

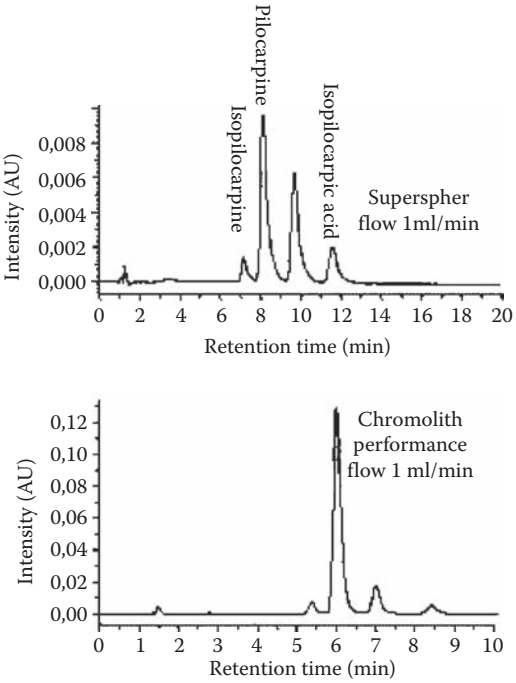
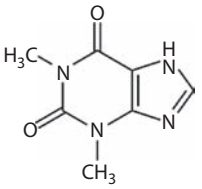
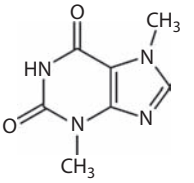


FIGURE 32.12 Representative chromatogram for pilocarpine hydrochloride and its degradation products on a conventional (Superspher RP-18e) and a monolithic (Chromolith Performance RP-18e) column. Mobile phase consisted of a buffer (pH 3)–methanol (98:2, v/v). (From ElDeeb, S., Preu, L., and Watzig, H., *J. Pharm. Biomed. Anal.*, 44, 85, 2007. With permission.)

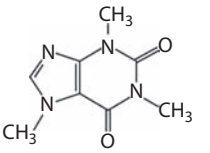
plants used as stimulant beverages, such as black and mate tea, coffee, cocoa, and soft drinks. It was reported that the plants of Sect. *Thea* contain the following compounds: caffeine, theobromine, theophylline, theacrine, adenine, xanthine, hypoxanthine, and paraxanthine and a small amount of pyrimidine alkaloids [110]. Caffeine appears as the major alkaloid (about 2–5%) in dry leaves of *C. sinensis* and *C. assamica*, which also contain small amounts of theobromine and theophylline [111]. Theobromine is the major alkaloid present (about 4–6%) in *Camellia ptilophylla*, which contains no caffeine or a small amount of it and a small amount of theophylline [112]. Theacrine is the major alkaloid present (about 1.3–3.4%) in *C. assamica* var. *kucha*, which also contains some caffeine and a small amount of theobromine and theophylline [113].



(22) Theophylline



(23) Theobromine



(24) Caffeine

These substances are widely known for their properties as stimulants of the central nervous system, respiratory system, skeleton, muscles, and heart. The mechanisms of action of caffeine and other methylxanthines are not well understood, but their main effects are due to the inhibition of phosphodiesterase, causing accumulation of cAMP. They may also block adenosine receptors [114]. Owing to their therapeutic properties (analgesic, diuretic, and bronchodilating), they have been utilized in many pharmaceutical formulations.

The International Olympic Committee (IOC) has classified caffeine as a drug of abuse when it is present in human urine in concentrations higher than 12 μg mL^{-1} [115]. Existing papers dealing with the determination of these compounds in urine for doping purposes [116–118] are based on liquid chromatography.

Several analytical methods have been proposed for determination of these methylxanthines in foods, pharmaceutical products, and biological fluids. Reversed-phase HPLC (RP-HPLC) has been most commonly used for this purpose (Table 32.6). Xanthine alkaloids, as very weak bases, are undissociated at the pH values commonly used in RP liquid chromatography; this is why they behave as neutral compounds. However, some of them dissociate as a weak acid. In this case, acids can be used to suppress dissociation, or a cationic pairing-reagent such as trioctylmethylammonium chloride can be used.

Most of the previously employed procedures were based on RP chromatographic separation with UV absorbance detection [116,124–126], MS detection [127], and amperometric detection [128]. The separation of these compounds could also be accomplished by ion-pair chromatography coupled with UV detection [129].

In plant material, purine alkaloids appear together with catechins and other polyphenols. Peng et al. elaborated a simple and fast HPLC analysis method for simultaneous determination of purine alkaloids and other compounds (phenolic compounds and theanine) in various *Camellia* species using an amide-C_{16} column. The limits of detection and quantification of the 14 compounds (catechins, gallic acid, caffeine, theobromine, theacrine, theophylline, and theanine) varied from 0.0001 to 0.072 ng/μL and 0.0004 to 0.24 ng/μL, respectively (Figure 32.13) [130].

Chen et al. [131] developed a method to separate caffeine, theobromine, and theophylline using ion-exchange chromatography either on a cation- or anion-exchange sorbent without any sample pretreatment. Also, Gennaro and Abrigo [129] developed a reversed-phase ion-interaction HPLC method with octylamine added to the mobile phase.

TABLE 32.6
Purine Alkaloids Determination

Aim of Analysis	Extraction Procedure	Separation System	Validation Parameters	Ref.
Purine alkaloids and catechins in tea from three typical tea plants: *C. sinensis*, *C. ptilophylla*, and *C. assamica* var. *kucha*	Precise, dry ground sample (0.50 g) was put into a 50 mL conical flask with a cap. Alkaloids and catechins were extracted with 40 mL boiling water for 30 min in a thermostated bath set at 90°C and shaken once every 20 min.	Mightysil RP-18 150 × 4.6 mm (5 µm) 5% (v/v) acetonitrile (solvent A) or 50% (v/v) acetonitrile (solvent B) containing 0.05% (v/v) phosphoric acid (85%). Solvent A maintained at 90% and solvent B maintained at 10% within the first 7 min. B increased linearly from 10% to 15% during 7–10 min, and this condition was maintained for 2 min. B increased linearly from 15% to 70% during 12–20 min, and this condition was maintained for 2 min. The flow rate was 1.0 mL/min and 10 µL was injected. The column temperature set at 40°C, and the monitored wavelength was 231 nm.	LOD (ng) Caffeine: 29.7966 Theobromine: 16.1181 Theacrine: 22.4433 The recovery of caffeine was 93.27%–95.04% at low level (about 1 mg), 93.36%–94.58% at midlevel (about 2 mg), and 96.54%–97.83% at high level (about 3 mg).	[119]
Catechins, gallic acid, and tea alkaloids (theobromine, theophylline, and caffeine) in tea samples	Tea sample (0.2 g) was extracted with 5 mL solvent (acetonitrile, water, methanol, aqueous methanol [15% and 70%] and acetone) with intermittent shaking (30 s on vortex mixer). The sample was then centrifuged at 1400 *g* for 10 min at 16°C. The supernatant was taken into a 10 mL volumetric flask, and the extraction steps repeated to reach the final volume of 10 mL.	C-18 Lichrocart column (250 × 4.0 mm, 5 µm). The mobile phase finally adopted was acetonitrile–0.1% *ortho*-phosphoric acid in water (w/v) with a flow rate of 1 mL/min and column compartment temperature of 35°C. Chromatograms recorded at 210 nm.	Standard curves for all standards except theophylline were plotted by injecting 2–10,000 ng/mL of standard mixture, and peak area responses were obtained. For theophylline, a standard curve was prepared by diluting the stock solution so that 0.1–200 ng/mL was injected into the HPLC. Obtained correlation coefficients were from 0.954 to 0.990.The coefficients of variation for the standard solution for all analytes were within 1.68%.	[120]

(*Continued*)

TABLE 32.6 (CONTINUED)
Purine Alkaloids Determination

Aim of Analysis	Extraction Procedure	Separation System	Validation Parameters	Ref.
Determination of theobromine, theophylline, and caffeine in cocoa samples	0.0100 g of powder was extracted with approximately 10 mL of hot water (80°C) in a heated circulating bath during 20 min. The solution was then cooled to room temperature and centrifuged at 3000 rpm, and the supernatant was filtered	C_{18} Nova-Pak column (150 × 3.9 mm, 4 µm) using a mobile phase consisting of a solution of 20% of methanol in water under isocratic conditions, at a flow rate of 1.4 mL/min. UV detected (274 nm).	Recoveries: > 95.0% LOQ: 0.50, 0.25, and 0.50 µg/mL for theobromine, theophylline, and caffeine LOD: 0.10 µg of theobromine/mL, 0.08 µg of theophylline/mL, and 0.10 µg of caffeine/mL The linearity of theobromine, theophylline, and caffeine to yield concentrations over the range from 2.0 to 20.0, from 0.5 to 10.0, and from 1.0 to 10.0 µg/mL, respectively	[121]
Methylxanthines (caffeine, theobromine, paraxanthine, and theophylline) in urine	To a 100 µL of urine sample in a 20 mL glass tube 900 µL of saturated ammonium sulphate solution was added, followed by a brief vortex mixing. About 7 mL of a chloroform–isopropyl alcohol (85:15, v/v) mixture was successively added, and the resulting mixture was vigorously shaken for 10 min, centrifuged at 2000 rpm for 10 min, and the organic phase carefully transferred into a tapered tube. The extract obtained was evaporated to dryness at room temperature under a gentle stream of nitrogen.	LC-18-DB column using 20:80 methanol–buffer (5 mM citric acid adjusted to pH 5 with triethylamine) Chromatograms were monitored at 280 nm	Recoveries ranging from 81.6 ± 2.6% (theophylline) to 99.3 ± 6.3% (theobromine) LOD and LOQ in urine ranged from 0.15 µg/mL (theophylline) to 0.3 µg/mL (theobromine) and from 0.8 µg/mL (theophylline) to 1.2 µg/mL (theobromine), respectively.	[116]

Methylxanthines (caffeine, theobromine, and theophylline) in urine, tea, and coffee	*Tea*: 1.47 g of tea was placed in infusion, in about 150 mL of water (approximately one cup), at 100°C for 3 min *Coffee*: 150 mL hot water/3.3 g coffee *Urine*: The urine samples (48 samples), obtained from volunteers, were stored at 4°C, defrosted before the analysis, and centrifuged in 10 min.	LiChrospher 100 RP-18 (244 × 4.4 mm ID, 5 µm; column linked to a LiChrospher guard column of similar characteristic (4 × 4 mm ID; Merck). Water–ethanol–acetic acid (75:24:1, v/v/v) as mobile phase and a flow rate of 1.0 mL min^{-1}. The detection was performed in UV at 273 nm.	LOD was 0.10 µg L^{-1} for caffeine, 0.07 µg L^{-1} for theobromine, and 0.06 µg L^{-1} for theophylline. LOQ was 0.23 µg mL^{-1} for theobromine, 0.18 µg mL^{-1} for theophylline, and 0.33 µg mL^{-1} for caffeine. Linearity 1.0–60 µg mL^{-1}. Spiked recoveries from the samples ranged from 94% to 105% for all analyses.	[122]
Determination of caffeine as a tracer of sewage effluent in natural waters (presence of caffeine in environmental waters)	The filtered sample was preconcentrated in a precolumn, which was backwashed with acidic water at pH of 2.70.	C_{18} column with a gradient of water–acetonitrile and detected by diode-array detection (DAD) at 210 nm. Four different precolumns: C_{18}, PRP-1, PLRP-s, and Env were evaluated for the on-line solid-phase extraction of caffeine.	Average recoveries were between 92.1±5.2% and 97.8±2.6%. Detection limits as low as 0.1 µg/L from 50 mL of sample were achieved.	[123]

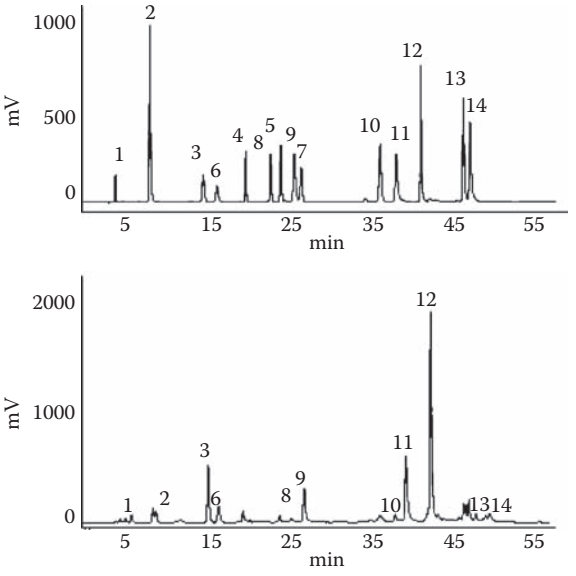


FIGURE 32.13 Chromatogram of standards and tea samples. (A) Standard; (B) Sample of *C. ptilophylla*. Conditions: Waters Symmetry C_{18} column (250 × 4.6 mm, 5 μm), mobile phase A: *ortho*-phosphoric acid–water (0.05:99.95, v/v); mobile phase B: acetonitrile. The gradient was as follows: 0–12 min, 5% B; 12–30 min, linear gradient from 5% B to 7% B; 30–40 min, linear gradient from 7% B to 20% B; 40–50 min, 20% B. Flow rate 0.8 mL/min. UV detection at 210 nm. Peak identification: (1) theanine, (2) gallic acid, (3) theobromine, (4) theophylline, (5) theacrine, (6) (–)-gallocatechin GC, (7) caffeine, (8) (–)-epigallocatechin EGC, (9) catechin, (10) (–)-epicatechin EC, (11) (–)-epigallocatechin gallate EGCG, (12) (–)-gallocatechin gallate GCG, (13) (–)-epicatechin gallate ECG, (14) (–)-catechin gallate CG. (From Peng, L., Song, X., Shi, X., Li, J., and Ye, C., *J. Food Compos. Anal.*, 21, 559, 2008. With permission.)

However, the great majority of the described methods need extensive sample pretreatment to reduce the interference from other compounds before the chromatographic determination. The extraction of methylxanthines can be performed using liquid extraction solvents such as dimethyl chloride, chloroform, and water [132,133]. However, this technique requires several hours to reach a complete extraction. Water, although an excellent solvent of methylxanthines, is highly nonselective, and its use may result in the removal of other valuable components from the extracted product, which gradually leads to deterioration of the analytical column [134]. In order to resolve this problem and improve the detection limit for environmental analysis, SPE was proposed for sample cleanup and preconcentration of caffeine using various cartridges [135–138].

Pura Naik [125] proposed the use of the Sep-pak C_{18} cartridge for the purification of cocoa extract before injection onto a HPLC C_{18} reversed-phase column. In this way, the interfering cocoa pigments are effectively removed. In the last decades, on-line column-switching devices, combined with precolumns packed with different kinds of materials, have been shown to provide a powerful and reliable solution for the on-line sample treatment of complex matrixes. It is therefore possible to inject the sample directly into the chromatographic system. Polyvinylpolypyrrolidone was packed in a precolumn and used to remove polyphenols from tea extracts on-line (Figure 32.14) [139,140]. Recently, automated systems have made it possible to perform SPE with a true extraction cartridge followed by desorption and further analysis on the analytical column in an on-line process. The determination of trace levels of caffeine (μg/L) in surface water and groundwater samples was possible in this way [123]. Spectrophotometric flow injection supported in liquid membranes was applied for determination of caffeine in solid and slurry coffee and tea samples [141].

Several studies have been reported determining tea alkaloids separately using HPLC following either isocratic or gradient elution methods [142,143]. Sharma et al. described an efficient and simple

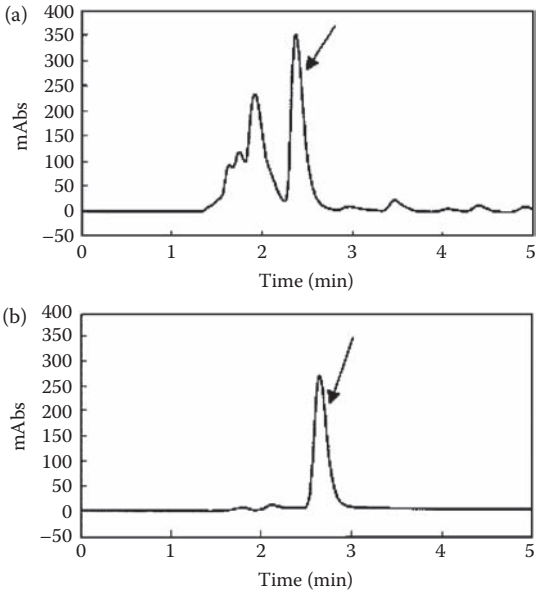


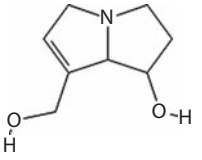
FIGURE 32.14 HPLC–UV chromatograms of tea extracts (a) without a PVPP precolumn and (b) with a PVPP precolumn. Arrow points to peak of caffeine. Analytical column (75 × 3 mm ID, 3 μm, ODS-UG-3, Nomura Chemical). The flow rate of the mobile phase (methanol–water–acetic acid, 40:59:1, v/v) was 0.6 mL/min. The wavelength of the detection was 272 nm. (From Horie, H., Nesumi, A., Ujihara, T., and Kohata, K., *J. Chromatogr. A*, 942, 271, 2002. With permission.)

gradient HPLC method for the simultaneous separation of tea alkaloids, catechins, and gallic acid in tea samples [120]. HPLC methods for simultaneous analysis of alkaloids and catechins have also been conducted by others for tea leaves of various varieties belonging to *C. sinensis* and *C. assamica* [139,144–148], *C. ptilophylla*, and *C. assamica* var. *Kucha* [119].

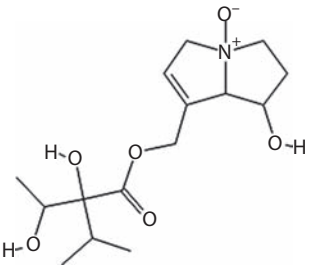
32.5.2 Pyrrolizidine Alkaloids

32.5.2.1 Senecio Alkaloids

Pyrrolizidine alkaloids (PAs) are secondary metabolites present in the genus *Senecio* that are derived biosynthetically from the amino acid ornithine. They are mainly distributed in the Boraginaceae, Compositae, and Fabaceae plant families. In nature, they occur as mono- or diesters of pyrrolizidine hydroxy derivatives (necines) or in the form of *N*-oxides. Taking into account their toxicity, PAs are naturally occurring carcinogens and can be classified in two groups: first, those possessing a saturated necine moiety in 1, 2-position, which are nontoxic substances, and second, those with an unsaturated necine base, which are hepatotoxic to mammals.



(25) Retronecine



(26) Indicine *N*-oxide

Isolation of pyrrolizidine alkaloids can easily be performed by SPE using C_{18} cartridges. This method gives good recoveries (> 90%) both for *N*-oxides and free bases [149,150]. Mroczek et al. proposed a sample-preparation method using a LiChrolut SCX (Merck, Darmstadt) polymeric strong cation exchanger, enabling very efficient isolation of both *N*-oxides and free bases of PAs, which could be further analysed by newly elaborated gradient ion-pair HPLC on a C_8 RP phase. Baseline separation of the alkaloids quantified in various plant-derived samples was achieved [151]. For the separation of pyrrolizidine alkaloids, RP phases such as C_{18} [152,153] C_8 [154], cyano-bonded [155], and amino-bonded phases [149] have been described. Due to the problem of peak asymmetry, ion-pair RP-HPLC is also advised [151].

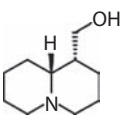
Identification of alkaloids is possible owing to hyphenated techniques. LC-MS analysis of macrocyclic PAs in *Senecio* species using a thermospray interface was reported [156]. Lin et al. [157] analyzed various types of macrocyclic PAs with both in-source (collision-induced dissociation in the collision cell) or out-source HPLC-MS or HPLC-MS/MS using an electrospray interface and quadrupole instrument. HPLC-APCI-MS was also reported for the determination of macrocyclic PAs [155]. Ndjoko et al. [156] applied the LC-NMR technique to distinguish *E* and *Z* isomers of senecionine in plant extracts. Mroczek et al. presented the on-line structure characterization of pyrrolizidine alkaloids (*N*-oxides and free bases) in two plant species (*Onosma stellulatum* W.K., family Boraginaceae, and *Emilia coccinea* Sims., family Compositae) performed using the newly elaborated RP-HPLC–ion trap MS method with APCI interface (Figure 32.15) [158,159]. In Table 32.7 methods of determination of pyrrolizidine alkaloids are collected.

32.5.3 Quinolizidine Alkaloids

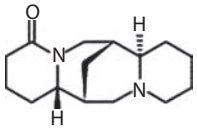
32.5.3.1 Lupine Alkaloids

Lupine alkaloids form a group of chiral bisquinolizidine compounds with a semirigid stereostructure. These are characteristic secondary metabolites of the Fabaceae. They are especially abundant in the tribus Genisteae, Sophoreae, and Thermopsideae. Within the Genisteae, members of the genus *Lupinus* rely substantially on quinolizidine alkaloids (QAs). Lupine seeds are especially rich in QAs. Lupine alkaloids are produced by leaf chloroplasts. They are distributed all over the plant via the phloem and stored in epidermal cells and in seeds. As a consequence, alkaloid profiles are more diverse in leaves than in seeds. Esters of 3β- and 13α-hydroxylupanine and of multiflorine are abundant in leaves whereas the hydroxylated alkaloids predominate in seeds.

Tetracyclic quinolizidine alkaloids with a sparteine, lupanine, and hydroxylupanine skeleton can be detected in almost all lupines. But the bicyclic lupinine, multiflorine, and aphylline and their derivatives, such as 10-oxosparteine-type, occur in a limited number of lupines. QAs with a α-pyridine skeleton such as anagyrine and cytrisine are typical for many genera of the Papilionoideae. As far as the toxicity of lupine alkaloids is concerned, lupanine- and sparteine-type alkaloids display a medium toxicity, whereas α-pyridine alkaloids such as cytisine are almost 10–100 times more toxic.



(27) Lupinine



(28) Lupanine

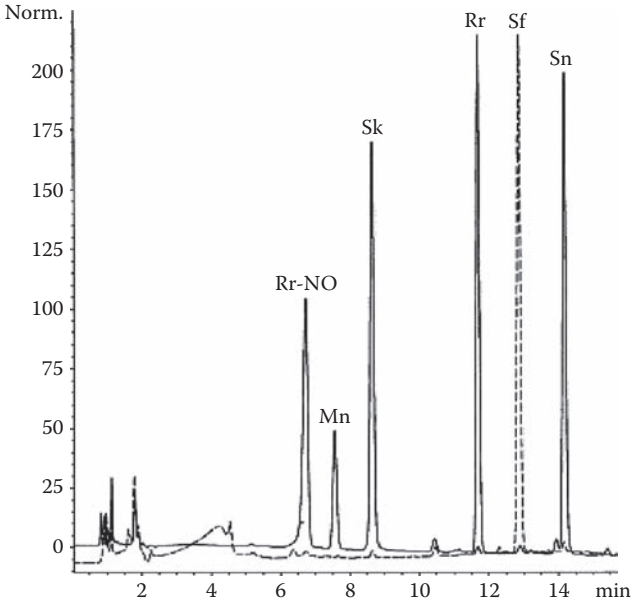
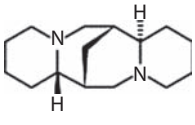
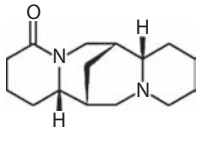


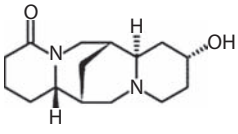
FIGURE 32.15 Reversed-phase HPLC separation of the alkaloid standards. Stationary phase: Waters XTerra C_{18}, 250 × 4.6 mm ID; d_p = 5 µm. Mobile phase: gradient of acetonitrile in 15 mM ammonia solution (see the text). Flow rate: 1.0 mL/min; column temperature: 25°C; injection volume: 10 µL. UV trace recorded at 220 nm. The concentrations injected: for retrorsine, retrorsine-*N*-oxide, senkirkine, senecionine, and seneciphylline, about 80 µg/mL, and for monocrotaline 40 µg/mL. Abbreviations used: Rr, retrorsine; Rr-NO, retrorsine-*N*-oxide; Sf, seneciphylline; Sn, senecionine; Sk, senkirkine; Mn, monocrotaline. (From Mroczek, T., Ndjoko, K., Głowniak, K., and Hostettmann, K., *J. Chromatogr. A*, 1056, 91, 2004. With permission.)



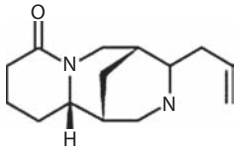
(29) Sparteine



(30) α-Isolupanine



(31) 13α-Hydroxylupanine



(32) Angustifoline

An alkaloid extraction procedure was proposed by Wink et al. [168]. According to this method, plant material was homogenized in 0.5 M HCl. After 30 min at room temperature, the homogenate was centrifuged for 10 min at 10,000 × g. The supernatant was made alkaline by adding ammonia or 2 M sodium hydroxide and was applied to Extrelut columns. Alkaloids were eluted with CH_2Cl_2 and the solvent evaporated in vacuo. An example of chromatographic fingerprints of the total alkaloids from *Caulophyllum robustum* is presented in Figure 32.16 [169].

TABLE 32.7
Pyrrolizidine Alkaloids Determination

Source of Alkaloids	Aim of Analysis	Method of Separation and Detection	Ref.
Gynura segetum (Lour.) Merr. (Jusanqi)	Pyrrolizidine alkaloids (PAs) and their corresponding *N*-oxides (PANOs). A total of 20 compounds were identified or tentatively characterized based on their mass spectra.	HPLC coupled to ion trap mass spectrometry (IT-MS)	[160]
Senecio scandens and *Tussilago farfara*	Pyrrolizidine alkaloids and their *N*-oxides	Micellar electrokinetic chromatography Direct-infusion electrospray ionization mass spectrometry and HPLC–electrospray mass spectrometry were used to analyze samples derived from liquid–liquid partitioning experiments and from strong cation-exchange solid-phase extraction of pyrrolizidine alkaloids and their *N*-oxides	[161]
Senecio scandens Buch.-Ham	Nine toxic and two nontoxic pyrrolizidine alkaloids were detected in the aqueous extract. Six of these (neoplatyphylline, senecionine, senecionine *N*-oxide, seneciphylline, seneciphylline *N*-oxide, and senkirkine) were unequivocally characterized, while other pyrrolizidine alkaloids were tentatively assigned as jacobine, jacozine *N*-oxide (or erucifoline *N*-oxide), 7-tigloylplatynecine, usaramine, and an isomer of yamataimine.	Aqueous extract of *S. scandens* collected in the Shanxi Province of China was determined, for the first time, to contain hepatotoxic and tumorigenic pyrrolizidine alkaloids by using HPLC–mass spectrometric analysis in various scanning modes	[162]
Senecio scandens	Retronecine esters-type pyrrolizidine alkaloids	Based on HPLC with prior derivatization of the alkaloids using *o*-chloranil and Ehrlich's reagent, chemical identity of the common retronecine marker was characterized on-line by positive-mode electrospray ionization mass spectrometry and nuclear magnetic resonance spectroscopy	[163]
18 plant samples from different collection sources and from different parts (flowers, leaves, thick stems, slim stems and roots) of *S. scandens*	Adonifoline, one of the retronecine-type hepatotoxic pyrrolizidine alkaloids	HPLC–tandem mass spectrometric analysis was conducted using a C_{18} column as stationary phase and a mixture of acetonitrile and aqueous formic acid as mobile phase	[164]

Crude extracts of *Senecio* species	Pyrrolizidine alkaloids. This approach led to the identification of the known pyrrolizidine alkaloids of *S. vulgaris*: retrorsine, seneciphylline, and senecionine	On-line coupling between HPLC, mass spectrometry, and ^{1}H-nuclear magnetic resonance The pyrrolizidine alkaloids present in the extracts were separated on a C18 reversed-phase column with an alkaline acetonitrile–water gradient.	[156]
Echium plantagineum	Pyrrolizidine alkaloids and their *N*-oxides	The subsequent integration of solid-phase extraction (with a strong cation exchanger) of the alkaloids and *N*-oxides from the aqueous acid solution, together with analysis using HPLC–electrospray ionization–mass spectrometry, provides a method for the simultaneous profiling of the pyrrolizidine alkaloids and their *N*-oxides in plant samples and the collection of useful structural data as an aid in their identification.	[165]
Honey samples	Pyrrolizidine alkaloids and their *N*-oxides	Honey samples were treated with methanol or dilute sulfuric acid and then centrifuged to remove insoluble material. Subsequent strong cation-exchange, solid-phase extraction of the supernatant provided a fraction that was analyzed for the presence of pyrrolizidine alkaloids and their *N*-oxides using HPLC coupled to electrospray ionization mass spectrometry.	[166]
Leaves of *Cerinthe minor*, *Cynoglossum clandestinum*, *Echium tuberculatum* (as well roots), *Eritrichium rupestre*, *Lithospermum purpureo-coerulem*, *Nonnea lutea*, *Nonnea setosa*, *Onosma stellulatum*, *and Cynoglossum amabile*	Pyrrolizidine alkaloids	Gradient HPLC with diode array detection (DAD) and thermabeam electron impact mass spectrometry (EI-MS). Dried plant material was extracted with boiling 1% tartaric acid in methanol for 2 h on an electric basket and crude extracts purified with cation-exchange solid-phase extraction. Purified extracts containing alkaloids were separated on a Zorbax SB RP18 stationary phase in a gradient of 0.1% formic acid in methanol.	[158]
Blood samples obtained from rats dosed with pyrrolizidine alkaloids and in the pyrrolizidine alkaloid–containing plant	Pyrrolizidine alkaloids	Collision induced dissociation (CID)-HPLC–mass spectrometry and HPLC–tandem mass spectrometry	[157]
Extracts of *Senecio jacobaea* (tansy ragwort) and *Senecio vulgaris* (common groundsel)	Separations of pyrrolizidine alkaloids and alkaloid *N*-oxide	Positive- and negative-ion thermospray liquid chromatography–mass spectrometry (LC-MS) with an ammonium acetate–containing mobile phase.	[167]

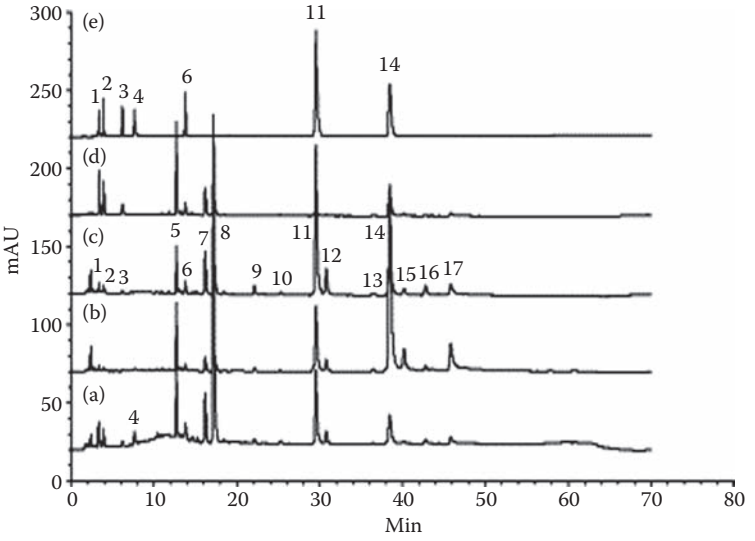
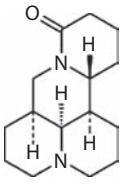


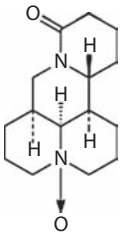
FIGURE 32.16 HPLC chromatogram of the total alkaloids from *Caulophyllum robustum*: (a) 220 nm; (b) 245 nm; (c) 267 nm; (d) 306 nm; (e) standard chemicals at 267 nm. Numbers 1–17 stand for peaks at 267 nm. Peak identification: 1. *N*-methylcytisine; 2. argentamin; 3. lupanine; 4. α-isolupanine; 6. anagyrine; 11. magnoflorine; 14. taspine. (From Yiping, L., Zhen, H., and Langchong, H., *J. Pharm. Biomed. Anal.*, 43, 1667, 2007. With permission.)

32.5.3.2 Matrine-Type Alkaloids

Matrine-type alkaloids are derived from a genus of Sophora including about 45 species of small trees and shrubs in the subfamily Faboideae of the pea family Fabaceae. Due to the high pharmacological activities of matrine-type alkaloids, the herbs have recently drawn specialists' attention in natural-medication research. Matrine (MT, 33) plays an important role in the treatment of tumors and arrhythmias. MT and oxymatrine (OMT, 34) also increase the leukocyte number and protect liver function. Matrine-type alkaloids can obviously inhibit varied clinical gastric mucosa damages, and enterocinesia. They have the effects of killing amoebas and Giardia lamblia stiles. It is indicated that matrine can be applied as a potential medicine for killing parasites. Studies have shown that some matrine-type alkaloids present positive myotome effects on cardiac muscle [170].



(33) Matrine



(34) Oxymatrine

Extraction is the most important part in the application of HPLC. Some extraction procedures, including liquid–liquid extraction (LLE), solid-phase extraction (SPE), or other methods can be applied for isolation of matrine-type alkaloids. These alkaloids, as weakly basic compounds

at a pH of about 9.5–11, can be extracted with chloroform. Simultaneously, most of them are soluble in acidic solutions. So, a two-phase solvent system composed of chloroform–methanol–water (4:3:2, v/v/v) acidified by phosphate buffer or hydrochloric acid is described [171]. Another method describes extraction by 95% ethanol. After the evaporation, the residue is dissolved with 2% hydrochloric acid. After filtration, the acidic aqueous solution, adjusted to pH 9.6 with sodium hydroxide, is extracted by chloroform [172]. The alkaloids in the plasma were extracted with chloroform–*n*-butyl alcohol (98:2, v/v) after basification and purification with neutral alumina SPEs [173].

Recently, molecularly imprinted polymers were employed as an SPE sorbent for chromatographic separation and the determination of matrine and oxymatrine from *S. flavescens* [174]. In this application, the SPE sorbent was obtained by the copolymerization of the cross-linker of poly (vinyl alcohol) and functional monomer of methacylic acid in the presence of template molecules. Matrine, as the template, was used for its preparative separation from Chinese medicinal plants. Methanol–water (3:7, v/v) was used for washing impurities from the molecularly imprinted polymer SPE cartridge loaded with the herb extracts, while methanol–glacial acetic acid (9:1, v/v) was used for eluting matrine. The maximum load of matrine and the recovery were 38.7 μg/g and 71.4%, respectively [174].

An attractive method for the preparative separation of matrine alkaloids is high-speed countercurrent chromatography (HSCCC), which provides, among other things, such advantages as a more than 10-fold increase in sample loading capacity, high-concentration fraction, and concentration of minor impurities [171]. Microwave energy has also been used for the extraction of matrine-type alkaloids by methanol–acetic acid (99:1, v/v). It should be stressed that the microwave extraction provides 20% higher efficiency than the shaken-flask extraction method, and decreases in time and solvent consumption can be achieved [173].

The most utilized technique for matrine-alkaloid separation is reversed-phase chromatography with UV detection at a wavelength of about 202–220 nm [175]. Besides the UV detection, in recent years, the electrochemiluminescent (ECL) method based on the chemiluminescence reaction of tertiary amine functional groups with Ru $(bpy)_3^{2+}$ has been applied for detection of matrine-type alkaloids [176]. The C_{18}-silica-based bonded phase is the most frequently used column. For incompletely endcapped silica-based phases, the ion-pairing or ion-suppression technique can be applied to avoid peak tailing. Micellar HPLC was achieved on a Phenomenex Tracar B 5 DOS (150 × 4.6 mm i.d.; particle size 5 μm) column with addition of 3.5% sodium dodecyl sulfate (SDS) to the mobile phase containing methanol–water (7:2) [177]. Furthermore, the addition of a little triethylamine to the mobile phase increases the peak symmetry and sharpness. However, the retention times of the alkaloids decrease with the increase in the amine concentration. Nevertheless, the most advantageous system appears to be ether-linked phenyl reversed-phase columns eluted with a gradient of aqueous methanol without any additives. The HPLC separation was performed also on amino-bonded phases. Separation of matrine alkaloids was performed on a Lichrosorb NH_2 column using acetonitrile-ethanol-*ortho*-phosphoric acid (85%) (80:10:8, v/v/v, pH 2.0).

Taking into account the hyphenated techniques, most quinolizidine alkaloids can be identified by LC-MS with atmospheric-pressure chemical ionization (APCI) and electrospray ionization (ES) as ionization methods. However, this technique is useful mainly for the analysis of less volatile quinolizidine alkaloids or their glycosides and volatile ion-pair reagents added to the mobile phase [178]. Typical chromatograms of standards and samples containing these alkaloids are shown in Figure 32.17 [179]. Table 32.8 lists methods for determination of matrine-type alkaloids.

32.6 DITERPENE ALKALOIDS

The *Aconitum* alkaloids comprise three kinds of highly toxic diterpene alkaloids—aconitine, mesaconitine, and hypaconitine—and their respective hydrolyzed monoester analogues benzoylaconine, benzoylmesaconine, and benzoylhypaconine.

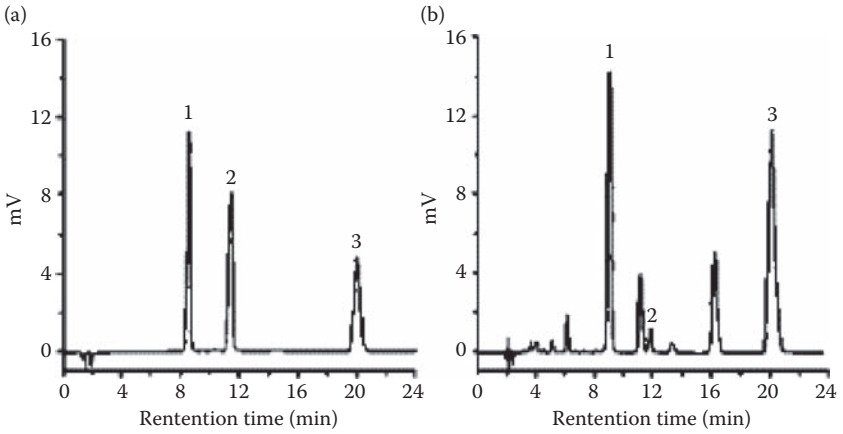
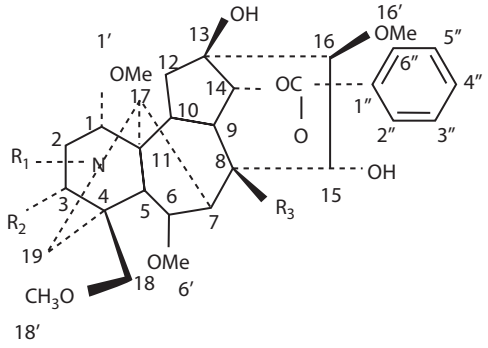


FIGURE 32.17 Chromatograms of (A) a mixture of matrine, sophoridine, and oxymatrine; and (B) *Sophora flavescens* Ait. Samples collected in Anhui province, China. (1) Matrine (9.4′); (2) sophoridine (12.2′); (3) oxymatrine (20.8′). HPLC separation of the alkaloids was performed on a Kromasil C_{18} column and detected by UV absorbance at 208 nm. The column temperature was maintained at 40°C. Mobile phase composed of 0.01 mol/L KH_2PO_4 buffer–methanol–triethylamine in the ratio 94:6:0.01 (v/v). (From Li, K. and Wang, H.J., *Biomed. Chromatogr.*, 18, 178, 2004. With permission.)



	R_1	R_2	R_3
1. Benzoylmesaconine	Me	OH	OH
2. Mesaconine	Me	OH	OAc
3. Aconitine	Et	OH	OAc
4. Hypaconitine	Me	H	OAc
5. Deoxyaconitine	Et	H	OAc

(35) The chemical structure of the *Aconitum* alkaloids

Because of their excellent effects against rheumatism and rheumatoid arthritis and some other inflammatory conditions, these medicinal herbs are widely used in clinics in China and other East Asian countries. The toxicology of *Aconitum* alkaloids derives from activation of the sodium channel of excitable cell membranes, leading to rapid paralysis of cardiac, neural, and muscular tissues. Therefore, identification and quantification of these toxic alkaloids in biological specimens is crucial in forensic medicine and therapeutic drug monitoring. *Aconitum* alkaloids in the aconite roots and in body fluids and tissues have been quantified, among other means, by the use of HPLC [181–183].

An acidic aqueous buffer, such as phosphate buffer with tetrahydrofuran as the organic phase (>10%), was used in most HPLC methods, with addition of sodium hexanesulfonate [195] or $CHCl_3$. In recent years, alkaline buffers, such as ammonium bicarbonate, have been used for separation of alkaloids, which provided better resolution ability [185].

Combining chromatography with MS offers the advantages of both chromatography as separation method and MS as an identification method. This kind of hyphenated technique has been applied for analysis of herbal materials, especially Chinese medicinal products containing toxic ingredients. LC-MS methods have been used for the determination of *Aconitum* alkaloids in biological samples [186–189].

TABLE 32.8
Matrine-Type Alkaloids Determination Methods

Objective	Alkaloid	Extraction Procedure	Mobile Phase/Column	Aim of analysis	Ref.
Caulophyllum robustum	*Lupine* alkaloids	Powdered *Caulophyllum robustum* was refluxed with 95% ethanol three times. Following evaporation of the ethanol *in vacuo*, the aqueous residue was extracted with 1% hydrochloric acid three times overnight while being stirred. The combined acidic fraction was passed through a positive-ion-exchange resin column (Lanxiao, China) and was washed with 4% ammonia–water–ethanol until the eluate tested negative for alkaloids in the Dragendorff test. The total alkaloids were obtained following the concentration of the eluate *in vacuo*.	Column: Zorbax SB-C_{18} column (250 × 4.6 mm, 5 µm, Agilent, USA) coupled with Agilent C_{18} guard column (7.5 × 4.6 mm, 5 µm). Mobile phase: (A) H_3PO_4–H_2O (0.01:100) and (B) CH_3OH–CH_3CN (3:1) in the gradient mode as follows: 10% B at 0–5 min, 10%–30% B at 5–20 min, 30%–65% B at 20–70 min. Flow rate 1.0 mL/min. Temperature 30°C. DAD detection 200–594 nm	Fingerprints by means of HPLC–diode array detector (HPLC-DAD) and gas chromatography–mass spectrometry (GC-MS)	[169]
Clathrotropis glaucophylla (Fabaceae)	Quinolizidine alkaloids	Air-dried and ground material was macerated to exhaustion at room temperature with EtOH 75% or CH_2Cl_2, MeOH, and MeOH–H_2O (80:20). The concentrated EtOH extract (32.7 g) was suspended in HCl (0.1 N) and extracted with CH_2Cl_2 (three times). The acidic solution was brought to pH 9 with 25% NH_4OH and extracted with CH_2Cl_2 (four times). The remaining water extract was brought to pH 11 with 25% NH_4OH and again extracted with CH_2Cl_2 (four times). The two CH_2Cl_2 extracts were combined and evaporated to dryness, yielding a dark brown syrup (alkaloid extract 1: 750 mg). For primary fractionation the alkaloid extract was submitted to open column (CC) on silica gel or aluminium oxide, using mixtures of dichloromethane and methanol of increasing polarity as mobile phase or hexane–ethyl acetate–ethanol respectively.	Particular fractions were isolated by normal-phase HPLC on silica gel, using CH_2Cl_2–MeOH–25% NH_4OH of increasing polarity as mobile phase.	The isolation and structure elucidation were performed with the aid of chromatographic (thin-layer chromatography, HPLC, and column chromatography) and spectroscopic (UV and 1D/2D nuclear magnetic resonance) methods, and mass spectrometry.	[180]

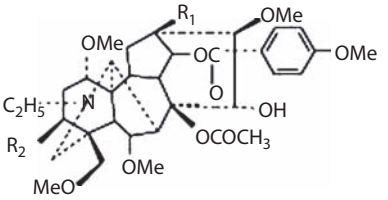
(*Continued*)

TABLE 32.8 (CONTINUED)
Matrine-Type Alkaloids Determination Methods

Sophora flavescens Ait	Matrine (MT), sophoridine (SR), oxymatrine (OMT)	Pulverized herbal sample (1 g) was extracted three times with water at 100°C for 2 h. The combined decoctions were centrifuged, and the supernatant was subjected to chromatography on a small column packed with Model 732 cation-exchange resins by eluting with water to remove the impurities and then with 50% alcohol to give the alkaloid fractions. The fractions were collected and transferred to a volumetric flask, diluted with the eluent. A 5 mL aliquot of the solution was transferred and evaporated to dryness in a water bath. The residue obtained was reconstituted in water and filtered through a 0.45 μm membrane.	0.01 MKH_2PO_4 buffer–methanol–triethylamine (94:6:0.01); Kromasil C_{18}	Linearity: MT, 0.2–120.0 μg/mL; SR, 0.2–115.2 μg/ml; OMT, 0.2–110.4 μg/mL Recovery: MT, 93.9; SR, 95.3; OMT, 93.5	[179]

Aconitum alkaloids are very unstable and decompose quickly in the human body (Figure 32.18). Some papers describe extraction, isolation, and identification of *Aconitum* alkaloids and their metabolites in human urine [190]. Aconitum alkaloids and their metabolites are separated and identified in human urine by LC-ESI-MS.

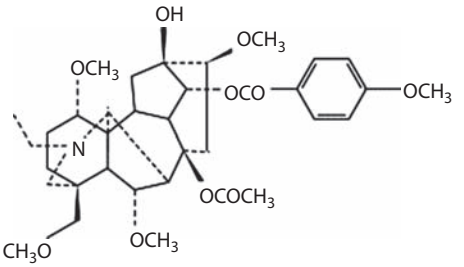
Most of the methods describe determination of *Aconitum* alkaloids containing the 8-*O*-acetyl-14-benzoyl functional group, such as aconitine, mesaconitine, hypaconitine, deoxyaconitine, and their semihydrolyzed derivatives. Rarely, yunaconitine, containing the 8-*O*-acetyl-14-anisoyl functional group, which is one of the main active and toxic constituents in many species of *Aconitum*, is detected [191]. Wang et al. described a new LC-MS/MS method combined with SPE, enabling detection of yunaconitine, together with crassicauline A and foresaconitine in urine [191].



	R_1	R_2
1. Yunaconitine	OH	OH
2. Crassicauline A	OH	H
3. Foresaconitine	H	H

(36) Structure of aconite alkaloids metabolites detected in human urine

Bulleyaconitine A, a diester–diterpene-type *Aconitum* alkaloid extracted from *Aconitum longtounense* is a potent analgesic and anti-inflammatory agent. The relative analgesic effect of bulleyaconitine A was found to be as much as 3 times as potent as 3-acetylaconitine and 65 times as potent as morphine. Only one paper has reported the analysis of bulleyaconitine A in biological fluids. There was established a rapid, specific, and sensitive method for the determination of bulleyaconitine A in human plasma using HPLC-MS/MS with the internal standard (I.S.) ketoconazole [192].



(37) Bulleyaconitine A

Table 32.9 presents methods of determination of *Aconitum* alkaloids.

32.7 POLYHYDROXY ALKALOIDS

Polyhydroxy alkaloids are natural products with different structures exhibiting potent activity as inhibitors of glycosidases. This group covers monocyclic and bicyclic alkaloids of the pyrrolidine, piperidine, pyrrolizidine, indolizidine, and nortropane classes, which possess two or more hydroxyl groups. As nitrogen-containing analogues of simple sugars, they also have similar glycosidase-inhibitory properties and can be applied as antiviral, anticancer, and antibiotic agents. The structural similarity to sugars and the inhibitory activity against glycosidase means that most polyhydroxylated alkaloids are antihyperglycemic. A piperidine alkaloid, 1-deoxynojirimycin (DNJ), is known to be one of the most potent β-glycosidase inhibitors. About 50 naturally occurring polyhydroxy alkaloids have been isolated from plants and microorganisms and identified [200–203]. Additionally, many synthetic analogues have also been prepared.

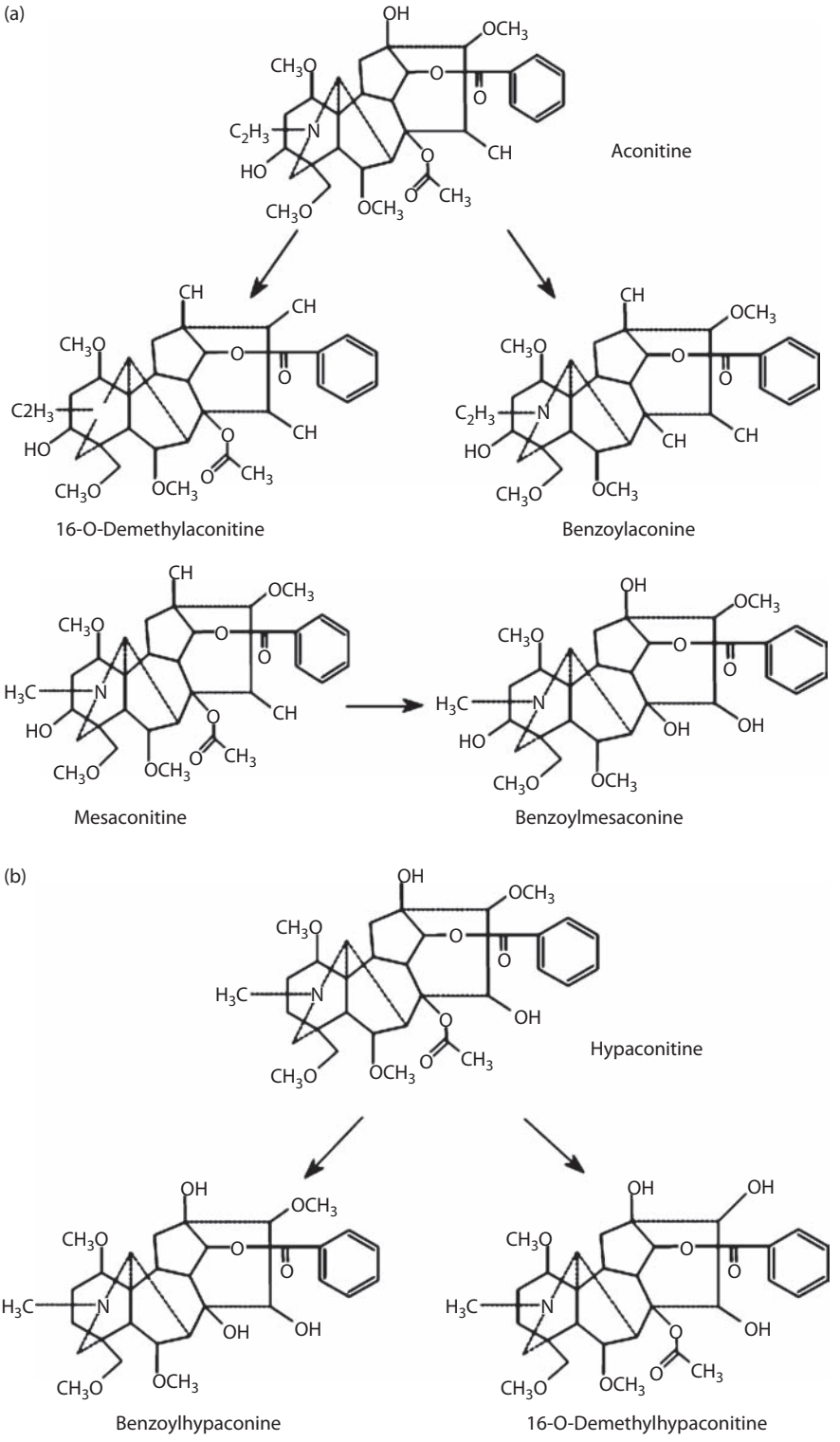


FIGURE 32.18 The structures of the *Aconitum* alkaloids and their metabolites. (From Zhang, H.G., Sun, Y., Duan, M.Y., Chen, Y.J., Zhong, D.F., and Zhang, H.Q.,*Toxicon*, 46, 500, 2005. With permission.)

TABLE 32.9
Determination of *Aconitum* Alkaloids

Sample	Extraction Procedure	Validation	HPLC Separation	Identification	Ref.
Determination of *Aconitum* alkaloids in blood and urine	SPE: blood samples (2 mL) were combined with four volumes of ACN–3% $HClO_4$ (4:96, v/v), and the supernatant was obtained by centrifugation. Urine samples (5 mL) were filtered through cellulose membranes. Deprotonized blood and filtered urine samples were applied to Sep-Pak cartridges that had been equilibrated with distilled water. After washing, the cartridge with 5 mL of 1 M HCl, alkaloids were eluted using a series of ACN–HCl solutions (5 mL). The eluate was evaporated and dissolved in 10 µL of methanol and diluted with 90 µL of water.	Recovery: Aconitine 94% Mesaconitine 100% Jesaconitine 98% Hypaconitine 100% Linearity: 2.5–500 ng Detection limit: 1 ng	Eluent: THF–0.2% TFA (14:86, v/v); column: ODS (150 × 4.6 mm) Detection: UV at 235, 260 nm. Temp. 40ºC	LC-frit-FAB-MS	[182]
Aconitine, mesaconitine, hypaconitine, benzoylaconine, benzoylmesaconine, and benzoylhypaconine in Chinese medicinal herbs, aconite roots	Each weighted sample was extracted with 10 mL HCl solution (0.05 M) by sonication for 60 min at room temperature, and then extracted with ethyl acetate for three times (10 mL each time) to remove nonalkaloid components. Then the acidic aqueous solution was basified with 28% ammonia solution to pH 10 and further extracted with chloroform three times (10 mL each time) by vortex-mixing for 2 min each. The resulting mixtures were centrifuged, and the combined supernatants were evaporated to dryness under air stream. The residue was further dissolved with 1.0 mL HCl solution (0.01 M) by sonication for 30 min.	Recoveries: 90%–103% Linear range (µg/ml): Benzoylaconine 2.48–248 Benzoylmesaconine 0.817–81.7 Benzoylhypaconine 2.12–212 Aconitine 1.13–135 Mesaconitine 1.21–146 Hypaconitine 1.05–126 µg/mL	Alltima RP18 (250 × 4.6 mm ID; particle size 5 µm; protected by an Alltima RP18 guard column (7.5 × 4.6 mm ID) at room temperature. Eluent: (A) acetonitrile and (B) buffer solution (containing 10 mM ammonium bicarbonate, adjusted with 28% ammonia solution to pH 10.0 ± 0.2). Gradient elution: 20%–25% A in 0–10 min, 25%–34% A in 10–30 min, 34%–45% A in 30–67 min, 45%–60% A in 67–75 min.	DAD. Detection was carried out at 240 nm with the reference wavelength of 550 nm.	[193]

(*Continued*)

TABLE 32.9 (CONTINUED)
Determination of *Aconitum* Alkaloids

Sample	Extraction Procedure	Validation	HPLC Separation	Identification	Ref.
Quantitation of six *Aconitum* alkaloids: aconitine, mesaconitine, hypaconitine, benzoylaconine, benzoylmesaconine, and benzoylhypaconine in human plasma	Sample preparation was performed on SPE using Waters HLB (30 mg sorbent). The Waters HLB cartridge was first conditioned with 1 mL methanol followed by 1 mL deionized water before use. 100 µL plasma and 10 µL (200 ng/mL) lappaconitine solution as the internal standard (IS) were loaded on the cartridge. The elution was carried out with 1 mL 5% methanol–water (v/v) and 1 mL methanol. The methanol fraction was collected and evaporated to dryness under a stream of nitrogen at 40°C. The residue was reconstituted in 100 µL mobile phase, and 5 µL sample was injected into the LC-MS/MS system for the analysis.	0.1 to 1000 ng/mL LOQs were 0.1 ng/mL for all six *Aconitum* alkaloids. LOD was 0.033 ng/mL for all target compounds. Linearity:0.1–1000 ng/mL	Waters C_{18} column (1.7 µm, 2.1 × 100 mm) and a gradient elution of methanol and 0.1% formic acid–water was used as mobile phase.	LC-MS/MS system coupled with an ESI source that was performed in multiple reaction monitoring (MRM) mode.	[194]
Determination of five principal alkaloids (benzoylmesaconine, mesaconitine, aconitine, hypaconitine, and deoxyaconitine) found in four species of genus *Aconitum*	Powdered samples (100 mesh, 0.1 g) were extracted three times with 0.2 M HCl (2 mL) with ultrasonic batch at room temperature for 30 min. The liquid phase was filtered, and the residues were washed twice with 0.2 M HCl (1 mL). The liquid phase was combined and loaded onto an Oasis® MCX extraction cartridge previously conditioned with 3 mL methanol and 3 mL water. After washing the cartridge with 3 mL 0.1 M HCl, 1 mL methanol, and 3 mL 1% NH_4OH successively, the alkaloids were eluted with a 3 mL solution containing 5% triethylamine in methanol. The eluate was dried under nitrogen at 35°C, and the residues were constituted with 1 mL 0.1 M HCl. The obtained solution was filtered through a membrane filter (0.45 µm) prior to injection.	The recovery of the method was 94.6%–101.9%, LOD ranged from 15 to 30 ng ml^{-1} detected at 233 nm. All the alkaloids showed good linearity ($\gamma = 0.9999$) in a relatively wide concentration range, 0.47–650 µg/mL.	XTerra RP18 column (250 × 4.6 mm ID, 5 µm) protected by a guard column (20 × 3.0 mm ID). The eluents were (A) aqueous 0.03 M ammonium hydrogen carbonate, adjusted to pH 9.5 with concentrated ammonia and (B) 100% acetonitrile. The mobile phase was linearly gradient (A:B): 0 min, 70:30; 40 min, 62:38; 60 min, 55:45 (v/v). The analysis was monitored at 233 nm. Column temperature was 35°C.	The compounds were identified by comparing their retention time and UV spectra with those of standards.	[195]

Aconitine, mesaconitine, and hypaconitine in the raw lateral roots of *Aconitum carmichaeli* and eight generally available traditional Chinese medicines	Each ground crude *fuzi* sample powder (0.2 g) was sonicated in methanol (30 mL) at 25°C for 30 min. The mixture was then centrifuged at 3000 rpm for 5 min. The supernatant was collected, filtered through a 0.2 μm PVDF syringe filter, and concentrated to a final volume of 10 mL. An aliquot (1 mL) of the sample solution was mixed with an equal volume of the IS (amphetamine was used as internal standard) solution (2 μg/mL) prior to analysis	LODs for these three markers were 0.05, 0.08, and 0.03 ng/mL. The linearity range for the three marker compounds aconitine and mesaconitine to hypaconitine was 0.1–1000 ng/mL.	Gemini C_{18} column (3 μm, 150 × 2 mm, Phenomenex, Torrance, CA, USA), with a Phenomenex Luna Security Guard Cartridge C_{18} (5 μm, 4 × 2.0 mm ID). Phase A consisted of water and 0.005% TFA (v/v); phase B comprised ACN and 0.005% TFA (v/v). Column separation was performed at room temperature by means of a gradient elution program, which started with 20% of phase B and increased linearly to 25% of phase B over 3 min and then to 28% B linearly over 7 min. Subsequently, the column was washed with phase B for 2 min and then equilibrated with the initial 20% phase B for 10 min.	LC-ESI-MS^n	[196]
Quantification of three major aconitine-type alkaloids (aconitine, mesaconitine, hypaconitine) in the roots of *Aconitum carmichaelii* Debeaux	Extracts from the roots of *A. carmichaelii* were prepared with mixtures of MeOH–H_2O (3:2, 3:1, 9:1, 1:0) and MeOH–$CHCl_3$ (3:1, 1:1, 1:3). 0.5 g ground root was extracted at room temperature three times (15 min each) in an ultrasonic bath with 3 × 15 mL solvent. The combined extracts were filtered, concentrated and diluted to 2.0 mL with MeOH. For acidic extraction, 1% aqueous HCl solution was used. After 15 min sonication with 5.00 mL solvent, the extract was centrifuged, and the supernatant was further analyzed.	12.3–184.5 μg/mL for aconitine (**1**), 21.1–422.9 μg/mL for hypaconitine (**2**), and 41.5–829.5 μg/mL for mesaconitine (**3**).	Zorbax Eclipse XDB-C_8 4.6 × 150 mm, 5 μm). A gradient system of 10 mM ammonium acetate buffer (pH 8.9) and MeOH: 0 min, buffer–MeOH 60:40; 35 min, buffer–MeOH 5:95; 42 min, buffer–MeOH 60:40; 45 min. The alkaloids were detected at 233 nm.	LC-APCI-MS^n HPLC–DAD	[197]

(*Continued*)

TABLE 32.9 (CONTINUED)
Determination of *Aconitum* Alkaloids

Sample	Extraction Procedure	Validation	HPLC Separation	Identification	Ref.
Chinese medicinal products for the presence of toxic ingredient compounds: three C_{19}-diterpenoid alkaloids, two quinolizidine alkaloids, two indole alkaloids, and four bufadienolide steroids	About 0.5 g homogenized sample was accurately weighed into a 15-mL centrifuge tube and mixed thoroughly with 10 mL of the respective mobile-phase solution. The mixture was ultrasonicated to extract for 30 min and separated by centrifugation at 2500 rpm for 15 min. The supernatant clear extract was filtered through 0.45 mm membrane filter prior to LC-MS/MS determination.	Mesaconitine: Linearity: 0.5–20 mg/L LOD: 0.6 ng Recovery: 90.3% Aconitine: Linearity: 0.5–20 mg/L LOD: 0.4 ng Recovery: 87.3% Hypaconitine: 0.5–20 mg/L LOD: 0.5 ng Recovery: 87%	The aconitine alkaloids were separated on a 250 × 2.1 mm ID, 5 µm, Hypersil C_{18} column from Alltech, USA. The mobile phase used was acetonitrile–0.4% v/v acetic acid with 0.01% v/v tetramethyl ammonium hydroxide (50:50) at a flow rate of 200 mL/min.	LC-MS/MS	[198]
Tetrodotoxin (TTX) and *Aconitum* alkaloids and their metabolites, such as aconitine, mesaconitine, hypaconitine, jesaconitine, benzoylaconine, benzoylmesaconine, benzoylhypaconine, and 14- anisoylaconine, in serum	The extraction column employed was a Shodex MSpak PK-4A (hydrophilic copolymer gel containing *N*-vinylacetamide, 10 L4.0 mm-ID, ShowaDenko). The mobile phase used for both introducing samples to the trap column and washing out the serum matrices was 10 mM ammonium acetate at pH 7.0. The flow rate was set at 1.0 mL/min, and the column compartment was maintained at 408°C.	For aconitine, mesaconitine, hypaconitine, and jesaconitine, linear calibration curves were obtained up to 500 ng/mL and LOD ranged from 0.2 to 1 ng/ml. For benzoylaconine, benzoylmesaconine, benzoylhypaconine, and 14-anisoylaconine, linear calibration curves were obtained up to 500 ng/mL, and LOD ranged from 2 to 50 ng/mL. Recoveries from serum samples were within the range 78%–119% for all the compounds studied.	Analytical column: Eclipse XDB-C_{18} (150 L2.1mm-ID, 5 mm particle size, AgilentTech.). Gradient elution was done with a mobile phase of (A) 80% 10 mM ammonium acetate at pH7.0 and (B) 20% acetonitrile containing acetate. The 80:20 (A–B) composition was set at 0 min, and the percentage of B was increased to 70% at 7 min and to 80% at 14 min. Flow rate 0.25 mL/min. Total analysis time was 35 min.	LC-MS-ESI method coupled with a column-switching technique	[199]

Aconitum alkaloids, aconitine, mesaconitine, jesaconitine, hypaconitine and deoxyaconitine, and their hydrolysis products	SPE using Sep-Pak Plus PS-1 was also utilized to clean up and concentrate alkaloids in blood and urine samples	The calibration curves of these alkaloids were linear in injection amounts ranging from 5 to 500 pg, and their detection limits were 1 pg per injection.	Linear gradient LC elution by solvent mixture of acetonitrile and 0.3% trifluoroacetic acid	LC–frit-FAB-MS	[185]
Identification and quantification of the toxic alkaloids yunaconitine, crassicauline A, and for esaconitine inurine specimens	SPE using Oasis MCX cartridges. Standard solutions were spiked into urine. The spiked urine samples (2 mL) were acidified with 50 µL 5 M HCl and filtered through cellulose membrane filter (0.45 m). Urine samples were applied to Oasis MCX cartridge, which had been conditioned and equilibrated with deionized water. After washing the cartridge with 0.1 MHCl and methanol, successively, the alkaloids were eluted by using 5% ammonium hydroxide in 70% methanol. The eluate was evaporated to dryness under 40°C and dissolved in 100 µL of mobile phase. 10 µL of the sample was injected into the LC-MS system.	Yunaconitine: Linearity: 0.2–500 ng/mL, LOD: 0.03 ng/mL, LOQ: 0.15 ng/mL Crassicauline A: Linearity: 0.2–500 ng/mL, LOD: 0.05 ng/mL, LOQ: 0.2 ng/mL For esaconitine: Linearity: 0.2–500 ng/mL, LOD: 0.05 ng/mL, LOQ: 0.2 ng/mL	The mobile phase consisted of acetonitrile–10 mM ammonium bicarbonate, adjusted to pH 9.5 with concentrated ammonium hydroxide (50:50, v/v). Flow rate: 0.2 mL/min. The column: Xterra RP18, 3.5 m particle size (100 × 2.1 mm) with guard column Xterra RP18 (10 × 2.1 mm). Operated at 30°C.	LC-MS/MS	[191]

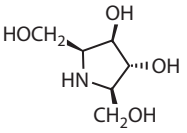
(*Continued*)

TABLE 32.9 (CONTINUED)
Determination of *Aconitum* Alkaloids

Sample	Extraction Procedure	Validation	HPLC Separation	Identification	Ref.
Determination of bulleyaconitine A (BLA) in human plasma	50 µL of internal standard (I.S.) ketoconazole solution (200 ng/mL) was added to 1.0 mL of each plasma sample. This mixture was extracted with 5 mL of ether by vortex for 1 min. The organic and aqueous phases were separated by centrifugation at 1500 × *g* for 10 min. The upper organic phase was transferred to another tube and evaporated to dryness at 35°C under a gentle stream of nitrogen. The residue was reconstituted with 100 µL of 0.1% acetic acid acetonitrile solution. The injection volume was 10 µL.	The extraction recoveries under the liquid–liquid extraction conditions were 76.35 ± 6.874%, 88.38 ± 5.931%, and 84.93 ± 5.048% at the concentration of 0.12, 1.2, and 6 ng/mL (QC samples), respectively. Acceptable linearity was observed over the range of concentration from 0.12 to 6 ng/mL for BLA in human plasma.	The chromatographic separation was performed using Waters Xterra MS C_{18} reversed-phase column (3.5 µm, 2.1 × 50 mm) equipped with a guard column of symmetry C_{18} (3.9 × 20 mm). The column temperature was maintained at 25°C. The mobile phase was composed of a premix of solvent A (0.1% v/v acetic acid aqueous solution) and solvent B (0.1% acetic acid acetonitrile solution). The mixture was eluted at 0.3 mL/min by a gradient method: 40% B from 0 to 1.5 min, 40% B to 95% B from 1.5 to 4 min, and 95% B from 4 to 6 min. The temperature of the sample cooler in the autosampler was 10°C.	HPLC-MS/MS	[192]

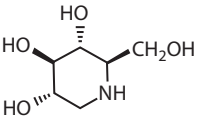
Note: APCI, atmospheric-pressure chemical ionization; DAD, photodiode-array detection; ESI, electrospray ionization; LC-frit-FAB-MS, capillary liquid chromatography (LC) fast atom bombardment mass spectrometry with a frit interface; LOD, limit of detection; LOQ, limit of quantitation; MS/MS, tandem mass spectrometry; MS*n*, multistage mass spectrometry; SPE, solid-phase extraction.

Pyrrolidine class



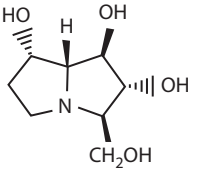
(38) 2,5-dihydroxymethyl-(DMDP)

Piperidine class



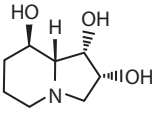
(39) 1-deoxynojirimycin (DNJ)

Pyrrolizidine class



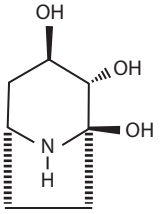
(40) Australine 3,4-dihydroxypyrrolidine

Indolizidine class



(41) Swainsonine

Nortropane class



(42) Calystegine A_3

Liquid–liquid Soxhlet extraction of plant material by 50–75% aqueous methanol or ethanol with addition of dilute acid is the first step [204], followed by SPE of the acidic portion onto anionic and cationic resins (Dowex 50 or Amberlite CG120) to purify the extract from other polar compounds (sugars and amino acids). Neutral compounds are eluted with water; nonbasic substances are displaced with 1–2 *M* pyridine, and polyhydroxy alkaloids are eluted with dilute ammonium hydroxide [205]. Particular alkaloids can be preparatively separated by collecting the fractions from the column [206]. Column chromatography on polar packing materials has been applied only rarely because the polyhydroxy alkaloids undergo very strong adsorption on such materials. However, swainsonine was separated by elution from silica gel with *n*-butanol–ethanol–chloroform–28% ammonium hydroxide (4:4:4:1) [207], and 1-deoxymannojirimycin was eluted from an alumina column with 90% ethanol [208]. Also, a trihydroxypyrrolidine was separated from a cellulose column by elution with *n*-butanol–pyridine–water (1:1:1) [205].

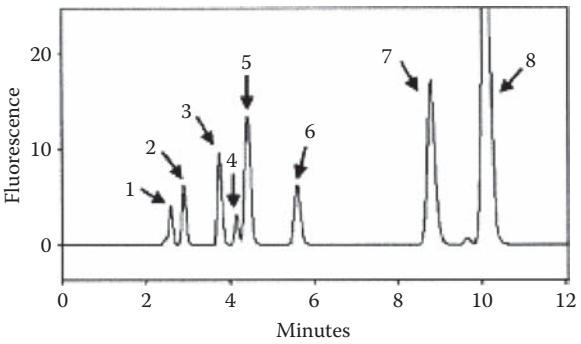


FIGURE 32.19 HPLC separation of 9-fluorenylmethyl chloroformate derivatized 1-deoxynojirimycin (DNJ-FMOC) (3) from seven other FMOC-labeled polyhydroxylated alkaloids in *M. alba*. (From Kim, J.W., Kim, S.U., Lee, H.S., Kim, I., Ahn, M.Y., and Ryu, K.S., *J. Chromatogr. A*, 1002, 93, 2003. With permission.)

TABLE 32.10
Polyhydroxy Alkaloids Separation Methods

Source	Stationary Phase	Mobile Phase	Detection	Validation Parameters	Ref.
Alkaloid mixture from seeds of *Castanospermum australe*	Cation-exchange packing (Partisil 10 SCX)	Acetonitrile–water (5:95) containing 0.015 N ammonium formate.	Thermospray LC-MS	Detection level for castanospermine, 500 pg	[212]
12 polyhydroxy alkaloids from different structural classes	Adsorbosphere XL Carbohydrate AX column, 150 × 4.6 mm ID, 7 µm	Acetonitrile–water gradient	LC-MS (APCI) and (ES)	Detection levels in positive-mode APCI were in the low picogram range and for ES at the nanogram level.	[213]
Swainsonine in different populations of locoweeds in the genus *Oxytropis*	Betasil C_{18} reversed-phase column, 100 × 2 mm ID	5% methanol in 20 m*M* aqueous ammonium acetate	APCI in the positive-ion mode with sequential tandem mass spectrometry (MS^2)	The detection limit of swainsonine was 0.019 mg/mL.	[214]
1-Deoxynojirimycin in *Morus alba* leaves after derivatization with 9-fluorenylmethyl chloroformate	Phenomenex Luna $C_{18}(2)$ column, 250 × 4.60 mm ID, 5 µm	Acetonitrile–0.1% aqueous acetic acid (1:1, v/v)	Fluorescence detector (excitation 254 nm, emission 322 nm)	Linearity ranged between 0.3 and 30 µg mL^{-1}	[210]

Abbreviations: APCI, atmospheric-pressure chemical ionization; ES, electrospray ionization; MS, mass spectrometry.

Structural identification of these compounds is possible owing to application of MS, NMR spectroscopy, X-ray crystallography, or circular dichroism (CD), which also is able to elucidate the stereochemistry, whereas for isolation, purification, and analysis, chromatographic methods can be applied [204,209].

The polar structure of polyhydroxy alkaloids results in their high-water solubility. Additionally, the lack of a chromophore permitting detection by UV absorption causes difficulties in their chromatographic analysis. Trimethylsilyl (TMS) derivatization followed by GC-MS has been the general method for the determination of polyhydroxylated alkaloids. Alternatively, liquid chromatography has also been considered. The resolution of closely related polyhydroxylated alkaloids without any of the chromophores needed for detections is difficult by LC. The detection problem could be surmounted by pre- or postcolumn derivatization of the hydroxyl groups with an appropriate chromophore or fluorophore. 9-Fluorenylmethyl chloroformate (FMOC-Cl) can be used for selective precolumn derivatization followed by RP-HPLC with fluorescence detection (Figure 32.19) [210].

The problem of detecting these alkaloids by HPLC analysis has been overcome to some extent by the use of a pulsed amperometric detector. MS possesses obvious advantages as an LC detector. Thermospray LC-MS, ESI, and APCI experiments have shown promising results for the analysis and identification of polyhydroxy alkaloids from plants and microorganisms. Negative-ion MS/MS, using an APCI source, has been demonstrated to be suitable for the identification of mono- and dihydroxypipecolic acid isomers and their respective epimers. LC–sequential MS (MS^n) and direct-infusion APCI-MS were used for identification of polyhydroxy alkaloids in *Hyacinthiodes non-scripta* [211]. Other examples of HPLC analysis of polyhydroxy alkaloids are collected in Table 32.10.

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Secondary Metabolites — Compounds Derived from Acetogenine (Acetylocoenzyme A)

33 HPLC Analysis of Polyacetylenes

Lars P. Christensen and Kathrine B. Christensen

CONTENTS

33.1 INTRODUCTION

Polyacetylenes belong to a class of molecules containing two or more triple bonds and constitute a distinct group of relatively unstable, reactive, and bioactive natural products. Polyacetylenes are widespread in nature, occurring in plants, fungi, lichens, moss, microorganisms, algae, and marine invertebrates [1–9]. The triple bond functionality of polyacetylenes makes these natural products a very interesting group of compounds whose reactivity toward proteins and other biomolecules may explain their wide variety of bioactivities.

Among the most interesting bioactivities of polyacetylenes are their antitumor and anti-inflammatory activity as well as their activity against fungi and gram-positive and gram-negative bacteria [8–12]. In particular, aliphatic C_{17}-polyacetylenes of the falcarinol type, which are widespread in common food and medicinal plants of the Apiaceae and Araliaceae plant families, are of interest. This type of polyacetylenes is highly cytotoxic and has a potential anticancer effect. In addition, they possess anti-inflammatory, anti-platelet-aggregatory, antibacterial, antimycobacterial, antifungal, neuritogenic, and immune-stimulatory effects, which may explain the beneficial effects associated with food and medicinal plants containing these compounds [9,10,12]. Fungi and microorganisms also provide a source for special polyacetylenes with interesting bioactivities that can be used to develop new antibiotics and anticancer agents [8,13].

Polyacetylenes are often unstable, being sensitive to ultraviolet (UV) light as well as to oxidative, thermal, and pH-dependent decomposition, which often leads to substantial challenges in their isolation, quantification, and characterization [1,9,12,14]. The most common

chromatographic methods for qualitative and quantitative measurements of these compounds in extracts and/or biofluids include high performance liquid chromatography (HPLC) combined with UV detection or mass spectrometry (MS) detection (LC-MS) and capillary gas chromatographic techniques (GC-flame ionization detector [FID] and/or GC-MS). A combination of column chromatography (CC) and HPLC enables isolation of these compounds from extracts in sufficient amounts and adequate purity for both characterization and testing in vitro and in vivo (animal models) for bioactivity.

This review introduces the distribution, biochemistry, and bioactivity of polyacetylenes isolated from higher plants and in addition gives an overview of the present state of knowledge on the analysis of polyacetylenes by HPLC.

33.1.1 Naturally Occurring Polyacetylenes in Higher Plants

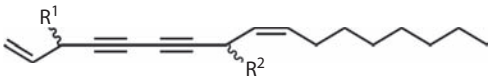
More than 2000 polyacetylenes have been isolated from natural sources, of which the majority are higher plants. Polyacetylenes are widespread among species within the botanically related plant families Apiaceae, Araliaceae, and Asteraceae, and in addition, they occur sporadically in at least 21 other plant families [1–9]. The polyacetylenes isolated from the Apiaceae and Araliaceae are characterized by aliphatic acetylenes, in particular C_{17}-polyacetylenes, whereas the structural diversity among the polyacetylenes from the Asteraceae, with more than 1100 polyacetylenes, is considerable and includes thiophenes, dithiacyclohexadienes (thiarubrines), thioethers, sulfoxides, sulfones, alkamides, chlorohydrins, lactones, spiroacetal enol ethers, furans, pyrans, tetrahydropyrans, tetrahydrofurans, and aromatic as well as aliphatic polyacetylenes. Polyacetylenes isolated from fungi, microorganisms, algae, and marine invertebrates include a wide variety of bioactive polyacetylenes including peptides, carotenoids, alkamides, and halogen-containing polyacetylenes as well as special long-chain aliphatic polyacetylenes [1,8,13].

The most common polyacetylenes in the Apiaceae are aliphatic C_{17}-acetylenes, in particular, falcarinol (**1**) and related polyacetylenes (**1–6**, **15–17**, and more; see Figure 33.1) [1,9,12,16–43]. In some genera, other aliphatic C_{17}-polyacetylenes are also typical constituents (Figure 33.2, e.g., compounds **18–32**), of which the majority are unrelated to polyacetylenes of the falcarinol type. Polyacetylenes of shorter chain lengths—primarily aliphatic C_{13}- and C_{15}-polyacetylenes derived from their corresponding C_{17}-polyacetylenes by oxidative degradation and oxidative transformation—also occur frequently in Apiaceae [1,9].

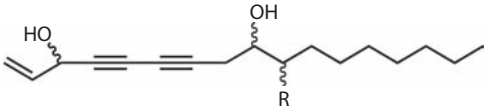
In the Araliaceae, the most common polyacetylenes are falcarinol (**1**) and related C_{17}-polyacetylenes (**2**, **6–14**, **17**, and more) of the falcarinol type, which confirms the close botanical relationship between the Apiaceae and the Araliaceae plant families. The isolation of dehydrofalcarinol (**33**) and related polyacetylenes (e.g., compounds **34–41**; see Figure 33.3) [44–49] from Araliaceae further connect this plant family to the Asteraceae.

In the Asteraceae, some types of polyacetylenes are widely distributed, whereas others are confined to specific tribes or genera. A characteristic feature for the majority of the aliphatic polyacetylenes occurring in the Asteraceae is the relatively high number of conjugated triple and double bonds in their structures, resulting in very characteristic UV spectra [1,14,43,50,51] making them easy to detect by HPLC combined with photodiode array (PDA) detection. The most common aliphatic polyacetylenes in the Asteraceae are C_{10}-, C_{13}-, C_{14}-, and C_{17}-polyacetylenes with an ene-diyne-diene, ene-diyne-ene, diyne-ene, triyne-diene, or triyne-ene chromophore but also compounds with four or five triple bonds in conjugation are widespread in most tribes of the Asteraceae (Figures 33.3 and 33.4) [1,3–8,10].

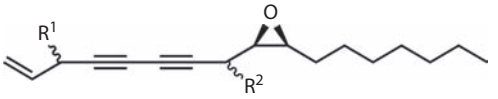
The aliphatic C_{17}-polyacetylenes in the Asteraceae include, beside the dehydrofalcarindiol type (Figure 33.3, e.g., compounds **33–36** and **41–45**), also a broad range of other C_{17}-polyacetylenes; examples of these are compounds **69–78** (Figure 33.4). Aliphatic C_{13}- and C_{14}-polyacetylenes (Figure 33.4, e.g., compounds **49–68**) are, together with C_{17}-polyacetylenes, the most widespread groups of polyacetylenes in this family [1,4,5,7,9].



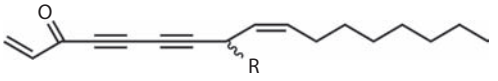
1 R^1 = OH, R^2 = H, Falcarinol (syn. panaxynol)
2 R^1 = R^2 = OH, Falcarindiol
3 R^1 = OH, R^2 = OCH_3, 8-*O*-Methylfalcarindiol
4 R^1 = OCH_3, R^2 = OH, 3-*O*-Methylfalcarindiol
5 R^1 = OAc, R^2 = OH, 3-*O*-Acetylfalcarindiol
6 R^1 = OH, R^2 = OAc, 8-*O*-Acetylfalcarindiol



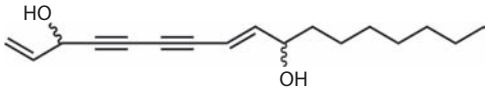
7 R = OCH_3, 10-*O*-Methylpanaxytriol
8 R = OAc, 10-*O*-Acetylpanaxytriol
9 R = OH, Panaxytriol



10 R^1 = OH, R^2 = H, Panaxydol
11 R^1 = OAc, R^2 = H, Acetylpanaxydol
12 R^1 = OH, R^2 = OCH_3, 8-Methoxypanaxydol
13 R^1 = OH, R^2 = OAc, 8-Acetoxypanaxydol
14 R^1 = R^2 = OH



15 R = H, Falcarinone
16 R = OH, Falcarinolone



17 Panaxydiol

FIGURE 33.1 Examples of aliphatic C_{17}-polyacetylenes of the falcarinol type, which are widespread within the Apiaceae and the Araliaceae plant families.

Polyacetylenic spiroacetal enol ethers are unique and characteristic for the tribe Anthemideae (Asteraceae). The five-membered C_{12}- and six-membered C_{13}-spiroacetal enol ethers are the most common (Figure 33.5, e.g., compounds **79–87**) [1,4,51–54], and some of these have shown interesting bioactivities, including anti-inflammatory and cytotoxic activities [1,4,51]. The tribe Anthemideae is also characterized by the presence of aromatic polyacetylenes, polyacetylenic lactones, alkamides, furans, hydrofurans, tetrahydropyrans, and sulfur compounds [1,4,51].

Aromatic polyacetylenes have been found primarily in plants belonging to the tribes Anthemideae, Cardueae, and Heliantheae (Figure 33.5) [1,4,5,7,9]. Phenylheptatriyne (**88**, PHT), present in all

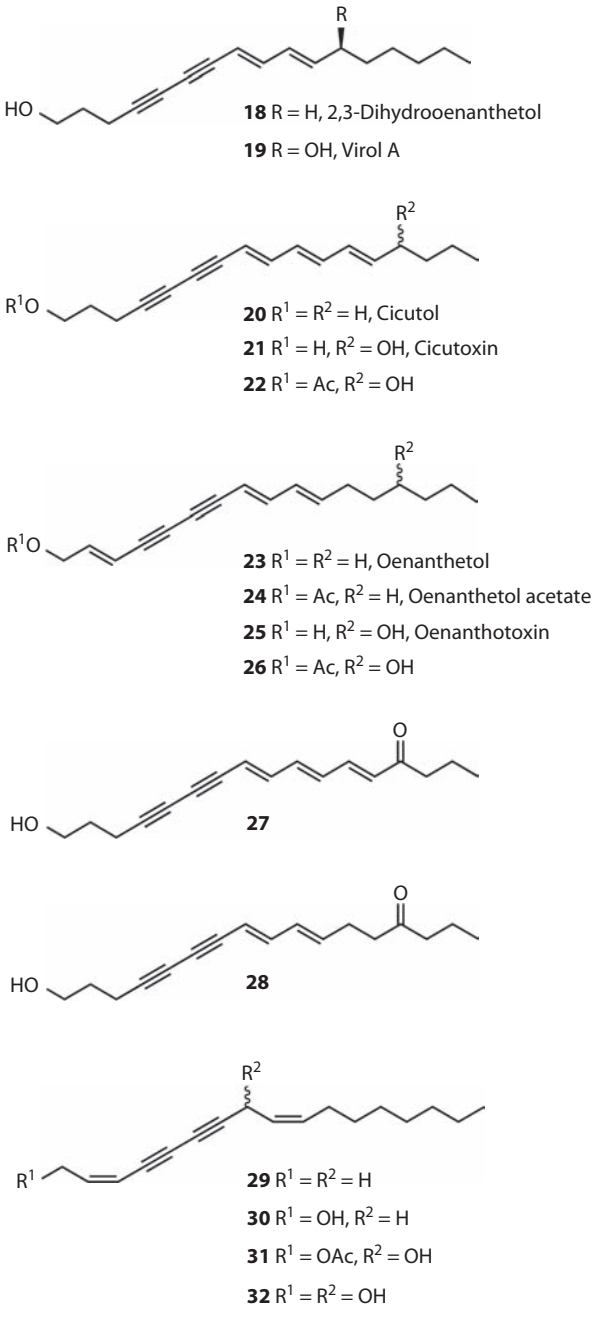
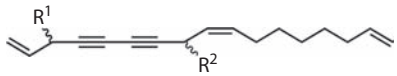


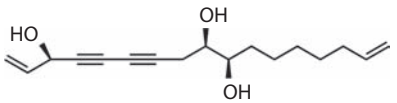
FIGURE 33.2 Examples of aliphatic C_{17}-polyacetylenes that occur in Apiaceae plant species and are unrelated to polyacetylenes of the falcarinol type.

three tribes, is highly phototoxic, while capillene (**89**), capillin (**90**), and related aromatic polyacetylenes isolated from *Artemisia* spp. (Anthemideae) [1,4,9,50,55–58] have demonstrated significant antifungal [10,59], anti-inflammatory [59–61], and cytotoxic activities [62,63]. Aromatic polyacetylenes such as **91** and **92** isolated from several *Argyranthemum* spp. (Anthemideae) also have cytotoxic and antimicrobial activity [64,65].

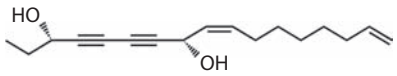


33 R^1 = OH, R^2 = H, Dehydrofalcarinol
34 R^1 = R^2 = OH, Dehydrofalcarindiol
35 R^1 = OAc, R^2 = OH, 3-*O*-Acetyldehydrofalcarindiol
36 R^1 = OH, R^2 = OAc, 8-*O*-Acetyldehydrofalcarindiol

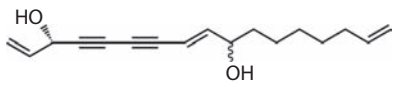
37 Ginsenoyne A



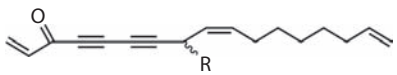
38 Ginsenoyne C



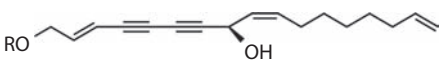
39 Dendroarboreol A



40 Dendroarboreol B



41 R = H, Dehydrofalcarinone
42 R = OH, Dehydrofalcarinonol
43 R = OAc



44 R = Ac, Gymnasterkoreayne D
45 R = H, Gymnasterkoreayne F

FIGURE 33.3 Examples of aliphatic C_{17}-polyacetylenes of the dehydrofalcarinol type occurring in several tribes within the Asteraceae but also seemingly characteristic for the Araliaceae.

Polyacetylenic tetrahydropyrans occur in the tribes Anthemideae, Cardueae, Heliantheae, and Inuleae [1,4,5,7,9]. The C_{14}-polyacetylenic tetrahydropyrans ichthythereol (**95**) and its acetate (**96**) display neurotoxic effects and were first isolated from *Ichthyothere terminalis* (Heliantheae), a plant species that has been widely used by native South American Indians as a fish poison [66,67]. Additional characteristic polyacetylenes isolated from Asteraceae are furanopolyacetylenes, tetrahydrofurans, and other *O*-heterocycles such as enol ethers (Figure 33.5). Furanopolyacetylenes have been found to occur regularly in the genus *Leucanthemum* (e.g., compound **99**; Anthemideae) [1,4,68,69] and in the genus *Atractylodes* (e.g., compounds **100** and **101**; Cardueae) [1,7,70–72] whereas polyacetylenic C_{13}-enol ethers such as compounds **102–105** are characteristic for the tribe Gnaphalieae [1,73–76]. Polyacetylenic tetrahydrofurans are rare in Asteraceae [1,4,5,7,9].

Sulfur-containing polyacetylenes are widely distributed within the Asteraceae. Besides in the Anthemideae they have been found to occur regularly in the tribes Arctoteae, Cardueae, Heliantheae,

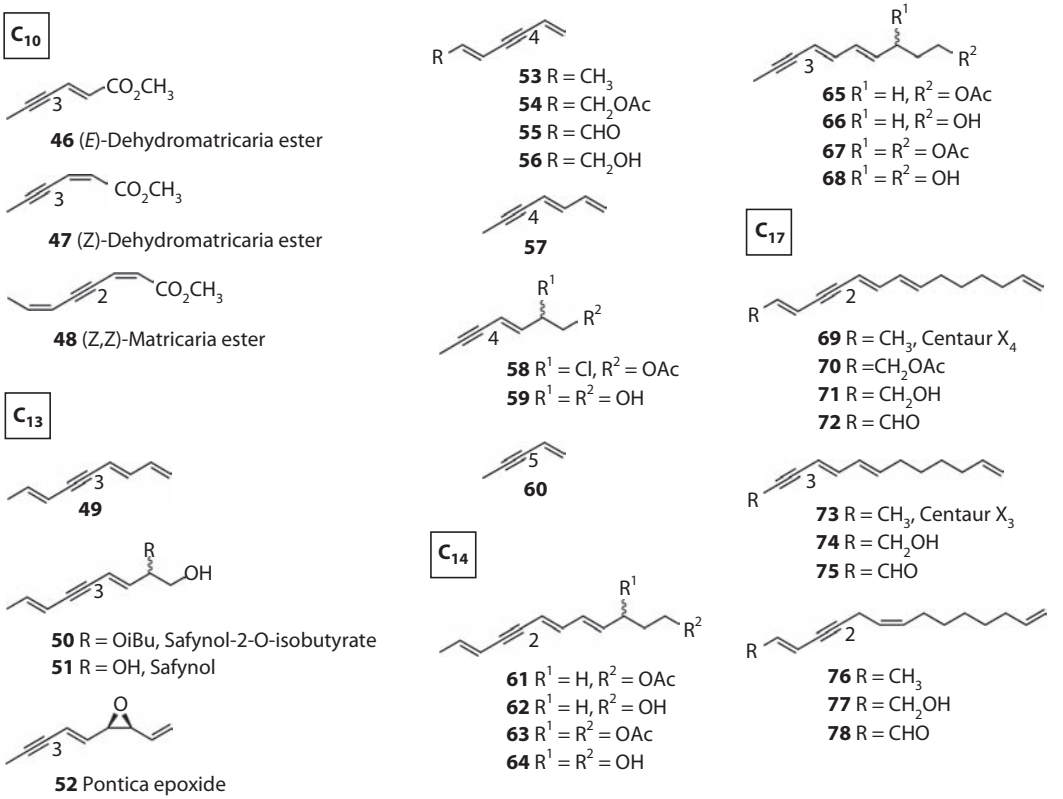


FIGURE 33.4 Examples of aliphatic C_{10}-, C_{13}-, C_{14}-, and C_{17}-polyacetylenes isolated from the Asteraceae plant species.

Inuleae, Mutisieae, and Plucheeae [1,3–5,7,9,77,78]. Mono- and dithiophenes constitute the largest and most widespread group of polyacetylenic sulfur compounds; examples of such polyacetylenic mono- and dithiophenes (**106–122**) are shown in Figure 33.6. The Anthemideae are also characterized by the occurrence of C_{10}-polyacetylenic methylthioethers such as compound **130** found in the genus *Anthemis* [1,4,79,80] whereas polyacetylenic C_{13}-methylthioethers, methylsulfoxides, and/or methylsulfones (Figure 33.6, e.g., compounds **131–133**) are primarily present in the tribes Heliantheae, Helenieae, and Inuleae [1,5,9,81–89]. From South African *Berkheya* spp. (Arctoteae), unusual thietanones (Figure 33.6, e.g., compounds **123–125**) have been isolated that seem to be characteristic for this tribe only [90–92]. Another special group of sulfur-containing polyacetylenes is the red dithiacyclohexadienes (1,2-dithiins), trivially known as thiarubrines (Figure 33.6, e.g., compounds **126–129**). Thiarubrines are restricted to certain genera within the tribes Heliantheae [1,4,93–102], Helenieae [1,4,103,104], and Inuleae [105]. Thiarubrine A (**126**) and other thiarubrines are antibiotics, and hence, many of the pharmacological effects of plants containing thiarubrines may be due to this type of compounds [102,106].

Polyacetylenic alkamides (Figure 33.7) have so far been found only in the Asteraceae, where they occur frequently in the tribes Anthemideae and Heliantheae [1,4,5,107,108]. In Anthemideae, the accumulation of polyacetylenic alkamides with up to three triple bonds is typical of the genus *Achillea* [1,3,4,107–112]. The genus *Achillea* comprises approximately 100 species and is especially rich in polyacetylenic pyrrolidides, piperidides, and the corresponding dehydro derivatives (pyrrolideides, piperideides). Most polyacetylenic cyclic amides found in this tribe consist of pyrrolidides (e.g., compounds **145** and **146**) and piperidides (e.g., compounds **142** and **147**; see Figure 33.7). In

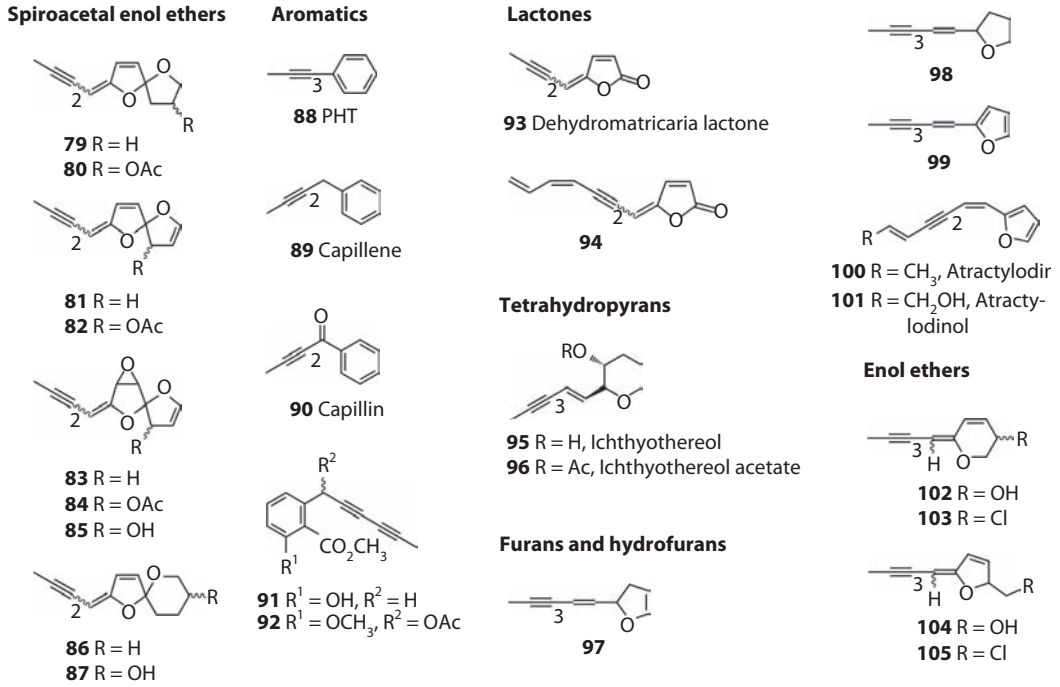


FIGURE 33.5 Examples of polyacetylenic spiroacetal enol ethers, aromatics, lactones, tetrahydropyrans, furans, hydrofurans, and enol ethers isolated from the Asteraceae.

Heliantheae, polyacetylenic alkamides are primarily found in the genera *Spilanthes* and *Echinacea*, where polyacetylenic isobutyl-, 2-methylbutyl-, and phenethyl amides (e.g., compounds **134**–**141**, **144**, and **148**–**150**) are common [1,3,5,107,108,113–124].

33.1.2 Biosynthesis of Polyacetylenes

The structural diversity observed among polyacetylenes seems to indicate the involvement of many different precursors in their biosynthesis. A comparison of the polyacetylene structures, however, clearly indicates that most polyacetylenes are biosynthesized from unsaturated fatty acids. Feeding experiments with ^{14}C- and ^{3}H-labeled precursors have confirmed this assumption and further that polyacetylenes are built up from acetate and malonate units [1–7,9,15,125–129]. The first step in the biosynthesis of polyacetylenes is the dehydrogenation of oleic acid and linoleic acid to the C_{18}-acetylene crepenynic acid (Figure 33.8). Two distinct pathways for the formation of acetylenic bonds have been proposed: desaturation of the existing alkene functionality through an iron-catalyzed dehydrogenation with molecular oxygen [128] or the elimination of an activated enol carboxylate intermediate driven by CO_2 formation [130]. The former pathway would be operative with full-length acyl lipids, whereas the latter would install acetylenic groups during de novo fatty acid biosynthesis [15,128,130].

Both hypotheses appear to be valid for the biosynthesis of polyacetylenes discussed in this review. Further dehydrogenation of crepenynic acid leads to diyne and triyne C_{18}-acids that, by α- and β-oxidation as well as other oxidative degradation reactions, lead to polyacetylene precursors of various chain lengths. These precursors are then transformed into a large variety of polyacetylenes. For example, the biosynthesis of falcarinol and related polyacetylenes follows the normal biosynthetic pathway for aliphatic C_{17}-acetylenes with dehydrogenation of oleic acid to give a C_{18}-diynic acid intermediate that is then transformed into C_{17}-diyne polyacetylenes by β-oxidation

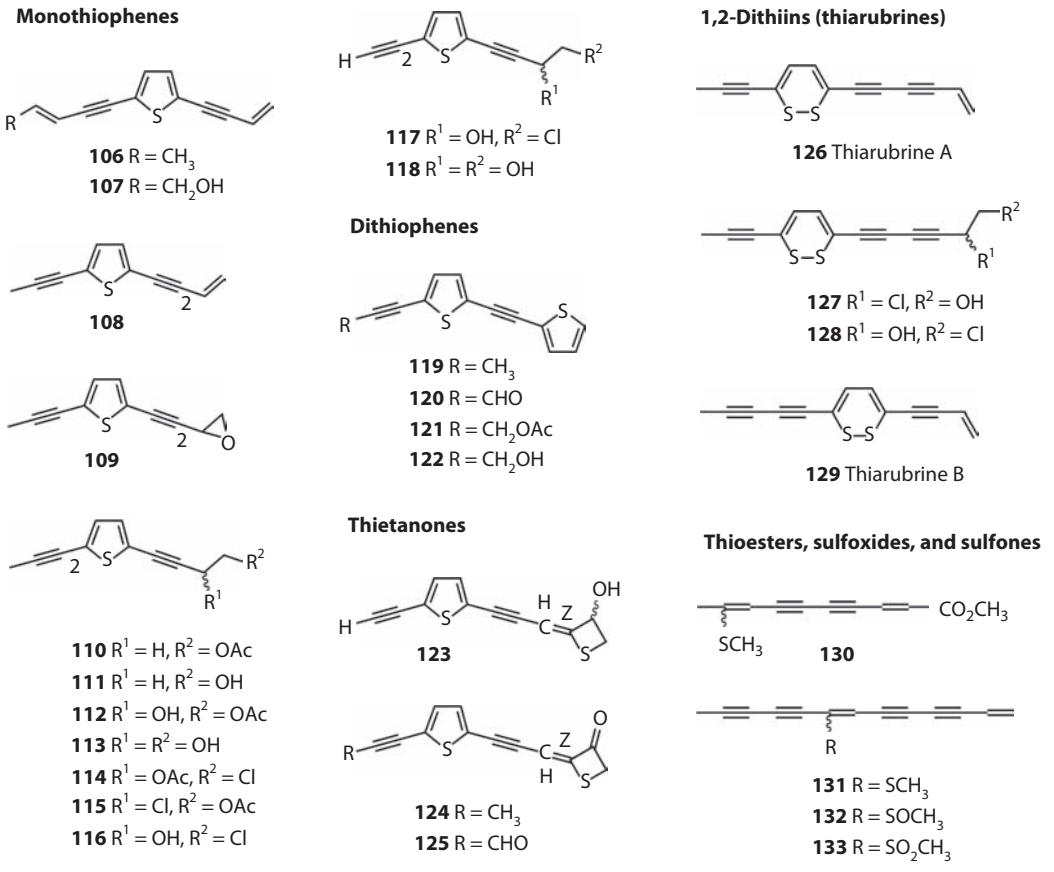


FIGURE 33.6 Examples of polyacetylenic mono- and dithiophenes, thietanones, dithiacyclohexadienes (thiarubrines), methylthioethers, methylsulfoxides, and methylsulfones isolated from the Asteraceae.

followed by the loss of CO_2 and H_2O [1,2,9]. Further oxidation and dehydrogenation then lead to polyacetylenes of the falcarinol type or the dehydrofalcarinol type, as outlined in Figure 33.8. More detailed information about the biosynthesis of polyacetylenes in higher plants as well as in lower plants, fungi, and microorganisms is available from several reviews on the subject [1–7,9,12,15].

33.1.3 Significant Pharmacological Effects of Polyacetylenes

Polyacetylenes are important for the pharmacological effects of many medicinal plants of the Apiaceae, Araliaceae, and Apiaceae as well as other plant families. Some of the most interesting pharmacological properties of polyacetylenes are neuritogenic, serotonergic, anti-inflammatory, antibacterial, antimycobacterial, antifungal, anti-platelet-aggregatory, and anticancer effects. In addition, the activity of some polyacetylenes is augmented by UVA light, where the primary mechanism of action seems to be either a photodynamic disruption of membranes, involving singlet oxygen, or a predominantly nonphotodynamic (oxygen-independent) mechanism of action [9,10,131–133]. The phototoxicity of polyacetylenes will, however, not be discussed further in this review.

The polyacetylenes cicutoxin (**21**) and oenanthotoxin (**25**) and related polyacetylenic derivatives (Figure 33.2, e.g., compounds **18–20**, **22–24**, **26**, and **27**) have neurotoxic effects as demonstrated in mice. Cicutoxin is the most neurotoxic of these polyacetylenes, with $LD_{50} < 3$ mg/

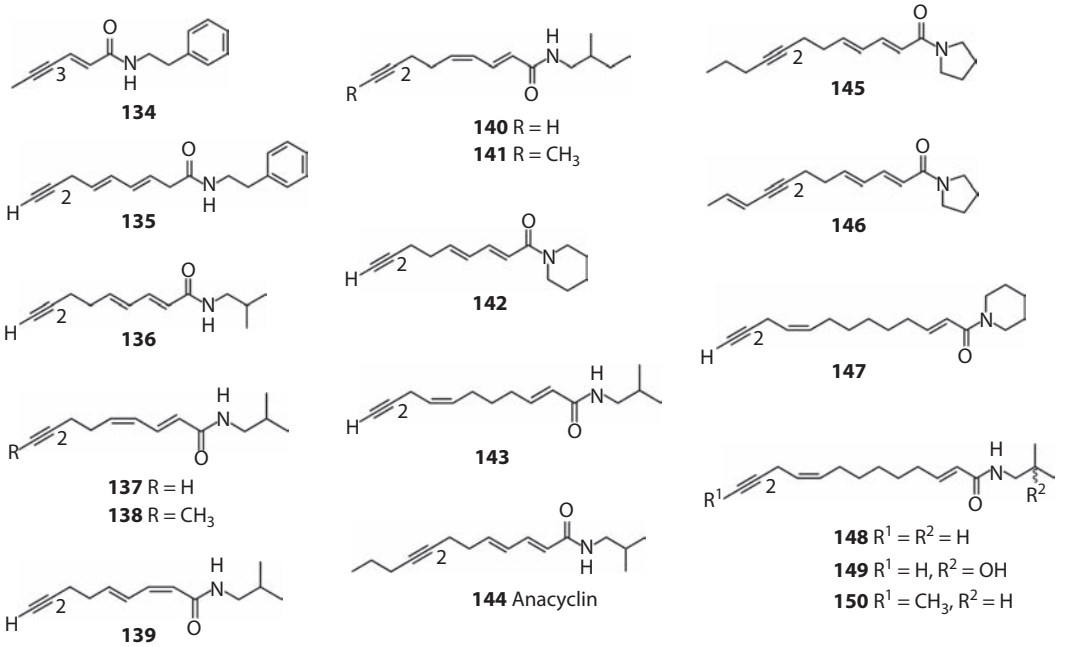


FIGURE 33.7 Examples of polyacetylenic alkamides isolated from the Asteraceae that occur frequently in certain genera of the tribes Anthemideae and Heliantheae.

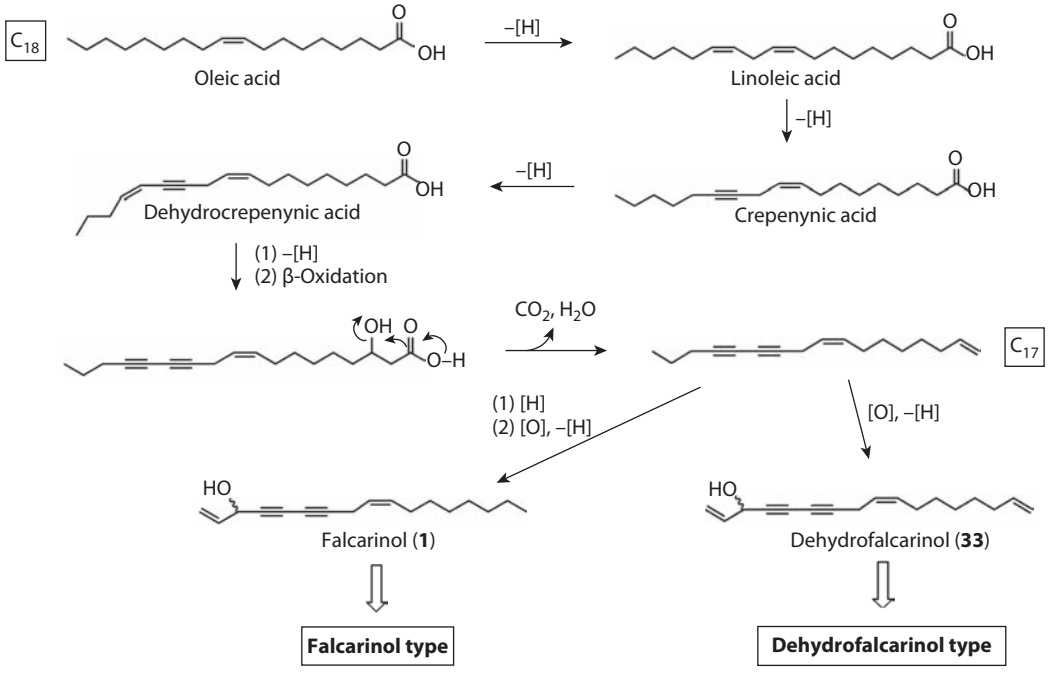


FIGURE 33.8 The possible biosynthesis of aliphatic C_{17}-polyacetylenes of the falcarinol and dehydrofalcarinol type. [O] = oxidation, [H] = reduction, –[H] = dehydrogenation (oxidation followed by the loss of water).

kg [24]. In comparison, C_{17}-polyacetylenes of the falcarinol type are much less neurotoxic than cicutoxin and related derivatives, with $LD_{50} > 100$ mg/kg for both falcarinol (**1**) and falcarindiol (**2**) [24,134]. However, falcarinol-type polyacetylenes affect neuritogenesis. For example, falcarinol and panaxytriol (**9**) have a significant neuritogenic effect on paraneurons like PC12h, PC12D, and Neuro2a cells in concentrations down to 2 μM [135,136]. Further, it has been demonstrated that falcarinol improves scopolamine-induced memory deficit in mice, which is probably due to its ability to promote neuritogenesis of paraneurons [135]. Falcarindiol and other falcarinol-type polyacetylenes may also act on serotonin receptors and thus exhibit pharmacological effects related to improvement of moods, behaviors, and other pharmacological effects mediated by serotonin [18].

Herbal remedies based on *Echinacea* species (*E. angustifolia*, *E. pallida*, and *E. purpurea*) are some of the best-selling herbal medicines in Europe and North America for the treatment of various infections [137]. Numerous bioactive compounds have been isolated from *Echinacea* spp., including alkamides, caffeic acid derivatives, and polysaccharides. Of particular interest is the anti-inflammatory activity of alkamides. Inhibition of 5-lipoxygenase (5-LOX) and cyclooxygenase isoforms (COX-1 and COX-2) has been demonstrated for several polyacetylenic isobutyl- and 2-methylbutylamides such as compounds **136**–**141** [116,138]. Some of these alkamides also display other forms of anti-inflammatory activity related to the production of macrophages [113].

Inhibition of 5-LOX and COX-1 activity has also been demonstrated among aliphatic polyacetylenes isolated from *Bidens camphylotheca*, of which safynol-2-*O*-isobutyrate (**50**) showed the strongest inhibitory effect (100% 5-LOX inhibition < 9.6 μg/mL; IC_{50} (COX-1) = 10.0 μM) [139]. Falcarinol and falcarindiol are also strong inhibitors of LOXs (5-, 12-, and 15-LOX) [140–144]. Furthermore, falcarindiol is an effective inhibitor of COXs, in particular COX-1, whereas the COX activity of falcarinol is not pronounced [140,144]. The anti-platelet-aggregatory effects of falcarinol and falcarindiol and hence, their protective effects against the development of cardiovascular diseases, are probably related to their anti-inflammatory activity, in particular, their ability to inhibit certain LOXs that are responsible for the production of thromboxanes, such as thromboxane B_2 [33,140,145,146]. Furthermore, it has been suggested that the anti-platelet-aggregatory activity of falcarinol is related to its ability to modulate prostaglandin catabolism by inhibiting the prostaglandin-catabolizing enzyme PGDH (15-hydroxy-prostaglandin dehydrogenase) [147]. Consequently, polyacetylenes of the falcarinol type may have a protective effect against the development of cardiovascular diseases such as atherosclerosis.

Extracts from *Plagius flosculosus* (Asteraceae) and *Daucus carota* (Apiaceae) have been shown to be able to inhibit the induction of the transcription factor NF-κB, which plays a key role in the inducible expression of genes mediating proinflammatory effects and thus is an important target for the development of anti-inflammatory agents. The anti-inflammatory agents responsible for these effects were identified as the spiroacetal enol ether **79** in *P. flosculosus* [54] and the falcarinol type polyacetylenes **1**, **2** and **5** in carrots [148].

Several polyacetylenes from the Asteraceae have shown an antibacterial effect against gram-positive bacteria (e.g., *Bacillus* spp., *Staphylococcus* spp., *Streptococcus* spp.) and gram-negative bacteria (e.g., *Escherichia* ssp., *Pseudomonas* ssp.) as well as antifungal activity against fungi such as *Candida albicans* and *Microsporum* spp. Polyacetylenes with bacteriostatic and/or fungistatic activity include aliphatic polyacetylenes (e.g., compounds **1**, **2**, **33**, **34**, **46–49**, **51**, **53, 60**), polyacetylenic spiroacetal enol ethers (e.g., compounds **79** and **82**), aromatic polyacetylenes (e.g., compounds **88–90**), and polyacetylenic thiophenes and thiarubrines (e.g., compounds **106**, **115**, and **126**) [9,10,20,39,59,60,131,132,149–161]. The antimycobacterial effects of falcarinol type polyacetylenes, such as falcarinol (**1**) and falcarindiol (**2**), toward *Mycobacterium* spp., including *M. aurum*, *M. fortuitum*, and *M. tuberculosis* [159,160], and antibacterial effects toward resistant strains of the gram-positive bacteria *Staphylococcus aureus* [20,159,161] are especially interesting. The antistaphylococcal activity, the effect against mycoplasma, and the other antibacterial effects occurred at approximately 10 μg/mL, that is, at nontoxic concentrations. Thus, this clearly indicates that falcarinol type polyacetylenes have positive effects on human health and may be used to develop new antibiotics.

Polyacetylenes of the falcarinol type (Figure 33.1) have cytotoxic activity and potential anticancer effects [9,10,12,13,45,46,162–173]. Falcarinol (**1**), panaxydol (**10**), and panaxytriol (**9**) are some of the most studied polyacetylenes and are highly cytotoxic toward cancer cells, including leukemia (L-1210), human gastric adenocarcinoma (MK-1), mouse melanoma (B-16), and mouse fibroblast-derived tumor cells (L-929) [164,166,167]. ED_{50} values toward MK-1 cancer cells have been reported as 0.027 μg/mL (**1**), 0.016 μg/mL (**10**), and 0.171 μg/mL (**9**) [167]. In addition, compounds **1**, **9**, and **10** inhibit the cell growth of normal cell cultures such as human fibroblasts (MRC-5), although the ED_{50} against normal cells was around 20 times higher than for cancer cells. In particular, panaxytriol did not even inhibit the growth of MRC-5 cells by 50% at concentrations above 70 μg/mL [167]. The apparent selective in vitro cytotoxicity of falcarinol type polyacetylenes against cancer cells compared to normal cells indicates that this type of polyacetylenes may be useful in the treatment of cancer. For example, the potential anticancer effect of falcarinol has recently been demonstrated in a preclinical trial in rats with azoxymethane (AOM)–induced colon preneoplastic lesions [174].

The mechanism of the cytotoxic activity and the potential anticancer effect of falcarinol and related C_{17}-acetylenes are still not known but may be related to their alkylating properties and hence, their ability to interact with various biomolecules [9,174]. Falcarinol has also shown apoptotic characteristics, which could be another mechanism of action of this type of C_{17}-polyacetylenes [9,173]. The cytotoxic activity of polyacetylenes of the falcarinol type and related polyacetylenes toward cancer cells and possible in vivo anticancer effect indicates that they may be valuable in the treatment and/or prevention of different types of cancer and could contribute to the health-promoting properties of medicinal plants as well as certain food plants.

Cytotoxic activities have also been demonstrated for polyacetylenes of the dehydrofalcarinol type and related C_{17}-polyacetylenes (Figure 33.3, e.g., compounds **33**, **34**, **36**–**40**, **44**, **45**) [9,10,45,46,168,169,175,176]. For example, the C_{17}-polyacetylenes from *Gymnaster koraiensis* (Asteraceae) exhibited significant cytotoxicity against L-1210 tumor cells with ED_{50} values as low as 0.12 μg/mL [175]. Also aliphatic C_{10}-polyacetylenes have been shown to possess cytotoxic and potential antitumor effects, which include the well-known (*Z*)- and (*E*)-dehydromatricaria esters (**46**, **47**) and their corresponding acids [177–179]. For example, (*Z*)-dehydromatricaria ester was able to inhibit the growth of cancer cell lines such as MK-1 (ED_{50} = 0.59 μg/mL), L-929 (ED_{50} = 0.98 μg/mL), and B-16 (ED_{50} = 1.87 μg/mL), making it almost as cytotoxic as C_{17}-polyacetylenes of the falcarinol type [179]. The potential anticancer activity demonstrated for aliphatic polyacetylenes of various chain lengths from the Asteraceae family is interesting and implies that other aliphatic polyacetylenes of this plant family may possess significant pharmacological activities yet to be discovered. Additional information about the cytotoxic activity and potential anticancer effect of polyacetylenes is available in recent reviews [9–13].

33.2 PHYSICOCHEMICAL CHARACTERISTICS OF POLYACETYLENES AND SAMPLE PREPARATION FOR HPLC ANALYSIS

Most polyacetylenes are thermally unstable and undergo photodecomposition if exposed to daylight. This instability is mostly prominent in condensed phases, where the stability depends on the distance between the polyacetylene molecules that are oriented in parallel. Furthermore, polyacetylenes are also sensitive to pH, being unstable in both acidic and basic environments [1,12,14]. Consequently, gentle methods should be used for extraction, detection, and isolation of these compounds. Polyacetylenes are extracted from plant material by an organic solvent such as *n*-hexane, diethyl ether (Et_2O), ethyl acetate (EtOAc), dichloromethane (CH_2Cl_2), or methanol (MeOH). Extraction of frozen plant material should be done when the plant material is still frozen to avoid enzymatic degradation of polyacetylenes and other secondary metabolites. The solvent used for extraction of polyacetylenes depends on the type of polyacetylenes to be analyzed. For polyacetylenes of high polarity such as glycosides and polyacetylenes with polar functional groups, MeOH is preferred. However,

when focusing on the lipophilic polyacetylenes that constitute the majority of the polyacetylenes isolated from higher plants, less polar solvents such as Et_2O or EtOAc are mostly used [9,12,14]. Extractions should be performed at 22°C or lower and always in the dark or in dim light to avoid degradation/decomposition of polyacetylenes during extraction. For the detection and quantification of polyacetylenes in extracts and/or fractions, thin-layer chromatography (TLC), UV spectroscopy, and HPLC combined with photodiode array (PDA) detection or simple UV detection are the most frequently used methods.

Analytical TLC is an excellent method for obtaining information on the number of polyacetylenes in extracts or fractions. In addition, TLC can give important information on optimal isocratic or gradient solvent systems for the separation of these compounds by column chromatography (CC) or HPLC. Normal-phase (NP) TLC is in general the best suited type of thin-layer plates for the separation of polyacetylenes, including polyacetylenic glycosides. Reversed-phase (RP) materials are widely used as stationary phases for the separation of polyacetylenes by HPLC, but are not widely used for the separation of polyacetylenes by TLC, probably because many polyacetylenes have a tendency to bind too strongly to the stationary phase due to their lipophilic character, resulting in tailing of the spots or bands [9]. Separation of polyacetylenes by NP TLC is usually performed on silica gel, where the typical adsorbent is Merck silica gel 60 F_{254}, 40–63 μm [9,12,14,180]. The possibility of using aluminium oxide (Al_2O_3) for separation of polyacetylenes has been investigated [9,14,181]. However, the high rate of decomposition has led to the abandonment of the use of Al_2O_3 for the separation of polyacetylenes by preparative TLC as well as for analytical TLC and CC.

For visualization of the spots on TLC plates, a number of spray reagents are used. A suitable spray reagent for the visualization of polyacetylenes on TLC plates is a neutral solution of potassium permanganate ($KMnO_4$, 0.32%), which is sprayed onto the plates. $KMnO_4$ oxidizes most organic compounds, leaving yellow spots [9,14,181]. For highly unsaturated compounds, such as polyacetylenes, the oxidation appears to be very fast in contrast to saturated compounds. Other visualization reagents that are used for detection of polyacetylenic compounds on TLC plates are vanillin, *p*-dimethylaminobenzaldehyde, and anisaldehyde dissolved in a solution consisting of ethanol (EtOH) and sulfuric acid (H_2SO_4) [9,14,122,182,183]. These visualization reagents give rise to spots with characteristic colors and can provide important information about the type or structure of polyacetylenes present.

For example, alkamides and other lipophilic constituents present in extracts of roots and/or aerial parts of *Echinacea* spp. are effectively separated by TLC on silica plates with *n*-hexane–EtOAc (2:1) as the mobile phase. The most prominent lipohilic constituents in *Echinacea* spp. are isobutyl- or 2-methylbutylamides, of which the majority are polyacetylenic alkamides, and 2-ketoalkenes and -alkynes [9,113–116,119,121–124,183]. Despite the complex mixture of constituents in plant extracts of *Echinacea* spp., in particular the roots, it is possible to distinguish between the individual species by TLC. Alkamides and 2-ketoalkenes and -alkynes are visualized using anisaldehyde/H_2SO_4 as a spray reagent. Alkamides with 2-monoenamide structures (e.g., compounds **143**, **148** and **150)** give characteristic yellow spots, while 2,4-dienamides (e.g., compounds **137–141**, and **153–155**) yield violet color reactions, except for the polyenic isobutylamides **151** and **152**, which result in blue-black spots. Hydroxylated polyacetylenic ketones (**156** and **157**) give dark gray spots, while 2-ketoalkenes or 2-ketoalkynes such as compounds **158–162** will result in yellow-brown color reactions (Figures 33.7 and 33.9) [9,122,183]. Further information about analytical and preparative TLC separation, visualization reagents, and characteristic color reactions of polyacetylenes is available in a recent review [9].

As polyacetylenes are highly conjugated substances consisting of at least two triple bonds in conjugation, they usually exhibit UV spectra with characteristic patterns between 200–410 nm and with high extinction coefficients (ε up to 350×10^3) at some wavelengths, typically below 380 nm [1,14,28,50,51,122,184]. Consequently, most polyacetylenes are very easy to detect and quantify in plant extracts by UV spectroscopy even at very low concentrations. The application of UV spectroscopy can therefore not only provide useful information about the structure

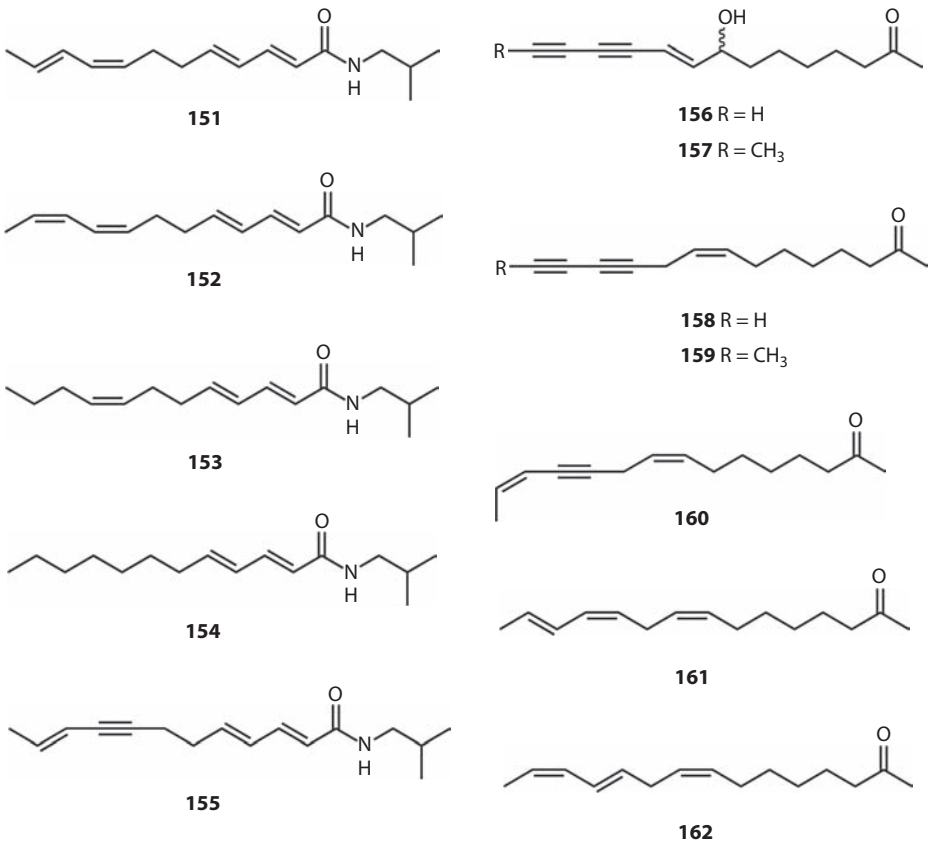


FIGURE 33.9 Chemical structures of some polyenic alkamides and polyacetylenic ketones detected in root extracts of *Echinacea* species by analytical thin-layer chromatography and HPLC (see Figures 33.17 and 33.18).

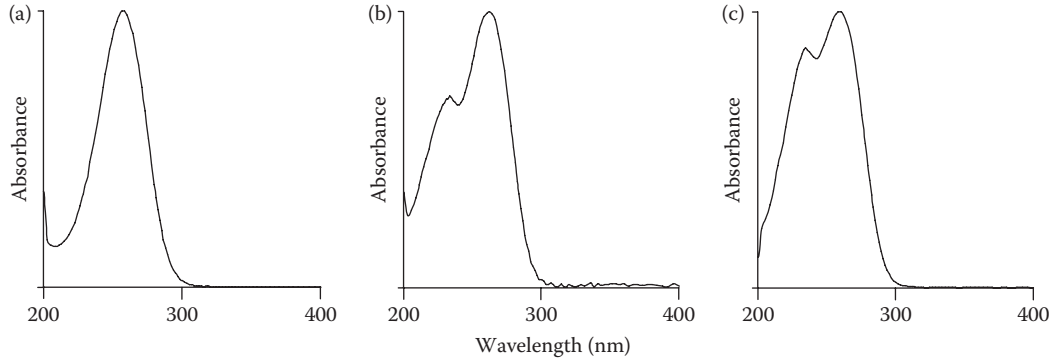


FIGURE 33.10 Characteristic UV spectra of polyenic and polyacetylenic alkamides detected in *Echinacea purpurea* root extracts. UV spectra obtained by HPLC–photodiode array detection for (a) compounds **137–141**, **153**, and **154;** (b) compounds **151** and **152;** and (c) compound **155**.

of the polyacetylenes, but also be useful for the detection and quantification of these compounds in samples/extracts [12,14,28,122,183,185]. Some examples of typical UV profiles of alkamides, and 2-ketoalkenes and -alkynes, occurring in *Echinacea* spp. are shown in Figures 33.10 and 33.11, and UV profiles of aliphatic polyacetylenes of the falcarinol type are shown in Figure 33.12.

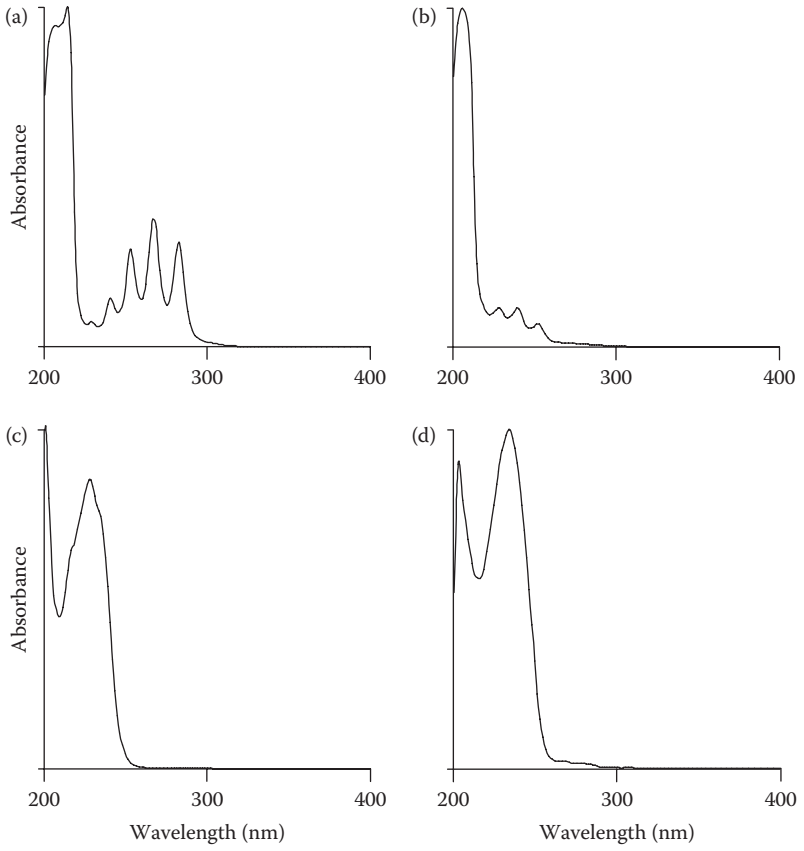


FIGURE 33.11 Characteristic UV spectra of polyenic and polyacetylenic ketones detected in *Echinacea pallida* root extracts. UV spectra obtained by HPLC–photodiode array detection for (a) compounds **156** and **157;** (b) compounds **158** and **159;** (c) compound **160;** and (d) compounds **161** and **162**.

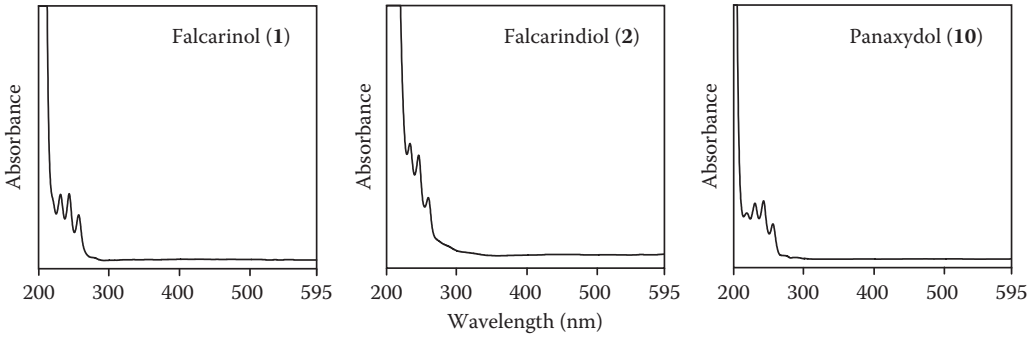


FIGURE 33.12 Characteristic UV spectra of some aliphatic polyacetylenes from carrot root (falcarinol, falcarindiol) and ginseng root (falcarinol, panaxydol) extracts obtained by HPLC–photodiode array detection. Absorption maxima: Falcarinol **(1)**: λ_{max} 231, 244, and 256 nm; Falcarindiol **(2)**: λ_{max} 233, 246, and 259 nm; Panaxydol **(10)**: λ_{max} 220, 231, 243, and 256 nm.

The following section comprises a description of some of the techniques and methods for detection, separation, and isolation of polyacetylenes in different types of samples/extracts. The application of HPLC for the analysis of alkamides and other polyacetylenes from *Echinacea* spp. and falcarinol-type polyacetylenes from carrot and ginseng roots are in focus.

33.3 PREPARATIVE AND SEMIPREPARATIVE HPLC

For determination of the exact chemical structure of polyacetylenes by spectroscopic means such as 1D- and 2D-nuclear magnetic resonance (NMR) techniques as well as for determination of the bioactivity of polyacetylenes, both in vitro and in vivo, a very high purity of the isolated compounds is required. For this reason, the use of a combination of CC and preparative and/or semipreparative HPLC is optimal, allowing isolation of large amounts of polyacetylenes at up to 99% purity. CC is usually the first step in the separation of polyacetylenes in plant extracts; the most used methods for separation of polyacetylenes are silica gel CC [9,12,14,19,28,58,186–191] or gel-permeation CC on Sephadex LH-20 [19,45,158,160,161]. For the separation of polyacetylenes by silica gel CC, flash CC is preferable [192], allowing separation of grams of extract into crude polyacetylene fractions within an hour or two. Moreover, the method has the advantage over conventional open-column chromatography that a small pressure of nitrogen protects against oxidation and hence, reduces the formation of artifacts during separation. The preferred column material for separation of polyacetylenes by flash CC is silica gel 60, 40–63 μm (230–400 mesh) from Merck [9,14,186–191].

The solvents used for development on the column are *n*-hexane or petroleum ether at the beginning, with an increasing amount of Et_2O, EtOAc, CH_2Cl_2, and/or MeOH [9,14,58,169,186–191]. For separation of polyacetylenes by gel-permeation CC on Sephadex LH-20, a mixture of CH_2Cl_2–acetone (85:15, v/v), 100% MeOH, or 100% EtOH are recommended [19,71,158,193]. To protect very unstable and light-sensitive polyacetylenes, it is preferable to stopper each fraction immediately after elution, protect it against UV light using tin foil, and finally store it in a refrigerator until analysis.

Isolation techniques such as CC, TLC, or more specialized techniques such as multilayer coil countercurrent chromatography, low-pressure liquid chromatography, and vacuum liquid chromatography used for the isolation of polyacetylenes are very time-consuming, especially if large-scale isolation and high purity are required [12,187]. These problems can be overcome using preparative or semipreparative HPLC as a final purification step. After separation of the polyacetylenes from the extracts by silica gel and/or Sephadex LH-20 CC, the polyacetylenes can be directly isolated from the crude fractions by preparative HPLC techniques [174,186,194,195]. The preferred preparative and/or semipreparative HPLC techniques for isolation of polyacetylenes are reversed-phase (RP)-HPLC using a simple stepwise gradient with an increasing concentration of aqueous MeOH or acetonitrile (MeCN) [174,186,194,195]. However, the use of other types of preparative and/or semipreparative columns, such as silica and aminopropyl-bonded, for large-scale isolation of polyacetylenes has been described [45,168,196]. Preparative or semipreparative RP columns such as ODS-Hypersil [21], Ultracarb-ODS [197], and ODS-HG-5 [174,186,194] are very efficient in separating polyacetylenes from crude polyacetylene fractions, as illustrated in the following examples by the isolation of falcarinol-type polyacetylenes at a purity above 98% from crude ginseng and carrot extracts by preparative HPLC.

Falcarinol type polyacetylenes have characteristic UV maxima above 225 nm (Figure 33.12). However, the detection wavelength for the analysis of these compounds by semi- and/or preparative HPLC-photodiode array (PDA) detection is usually 205 nm [12,19,186,194] in order to be able to detect nearly all types of compounds in the sample. This ensures an optimal separation and purification of the polyacetylenes. The isolation of relatively pure falcarinol (**1**) and panaxydol (**10**) from ginseng root extracts can be achieved by semi- and/or preparative RP-HPLC, even from very crude fractions, as illustrated in Figure 33.13. The collected polyacetylene fractions resulting from the separation by semi- and/or preparative HPLC are then analyzed by analytical

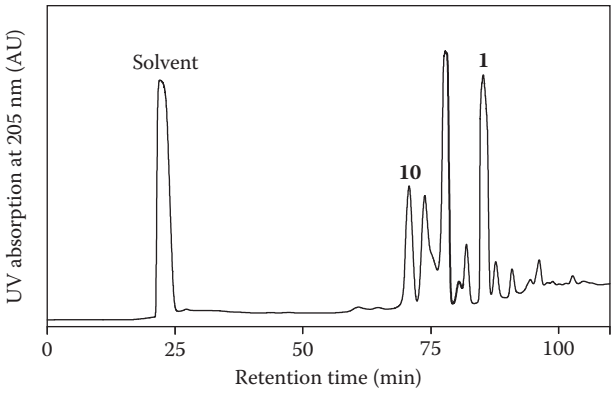


FIGURE 33.13 Preparative reversed-phase (RP)-HPLC chromatogram showing the separation of the polyacetylenes falcarinol **(1)** and panaxydol **(10)** from a crude polyacetylene fraction resulting from the separation of a ginseng extract by silica gel flash column chromatography. Solvent = ethyl acetate. Polyacetylenes were separated on a RP Develosil ODS-HG-5 HPLC column (RP-18, 250 × 20 mm ID, Nomura Chemical Co., Seto, Japan) at 25 ºC using the following solvent gradient: methanol–water, 0 min (40:60), 10–40 min (75:25), 80–110 min (100:0), 115 min (40:60). All increases/decreases in the gradient were programmed as linear. Flow rate: 5 mL/min. Injection volume: 25 mL. Detection: photodiode array (PDA) detector operating from 200–595 nm. Acquisition off at 110 min.

HPLC in order to check for impurities (see Section 33.4). From crude fractions as shown in Figure 33.13, it may be necessary to do an additional purification step by semi- or preparative HPLC, including optimization of the solvent gradient, in order to remove small amounts of impurities from the purified polyacetylenes. The result on the other hand is polyacetylenes of very high purity suitable for chemical characterization by NMR and other spectroscopic techniques and testing for bioactivity in vitro and in vivo.

Fractionation of extracts by silica gel CC, however, normally results in a relatively good separation of the individual polyacetylenes. For example, a good separation of polyacetylenes from EtOAc extracts of carrot roots by silica gel CC can usually be achieved using a solvent gradient of *n*-hexane and EtOAc [186,194], which may result in relatively pure polyacetylene fractions of falcarinol, 3-*O*-acetylfalcarindiol (**5**), and, in particular, falcarindiol (**2**). The small amount of impurities detected in a falcarindiol fraction obtained by CC from a carrot root extract was effectively separated from the falcarindiol peak by preparative RP-HPLC as illustrated in Figure 33.14. Purification of falcarindiol from this fraction by preparative RP-HPLC resulted in the isolation of falcarindiol (approximately 250 mg) as a colorless oil at a purity above 99% as determined by analytical HPLC, TLC, and NMR analysis (unpublished data). This example illustrates that polyacetylenes can be isolated in relatively high amounts and in a very high purity from crude extracts using a combination of CC and semi- and/or preparative HPLC.

Bioassay-guided chromatographic fractionation can be performed by CC, preparative TLC, and/or preparative HPLC, testing every fraction resulting from the fractionation in a relevant bioassay. As mentioned above semi- and preparative HPLC techniques are normally used as the final purification step to obtain bioactive compounds of high purity for chemical characterization and testing of bioactivity. However, semi- and/or preparative HPLC are also obvious techniques to use when searching for bioactive polyacetylenes and other secondary metabolites in crude extracts/fractions as long as one remembers to test all collected fractions resulting from the HPLC separation. For example, preparative HPLC has successfully been used to identify falcarinol, 3-*O*-acetylfalcarindiol, and falcarindiol as anti-inflammatory constituents of crude purple carrot root extracts using the HPLC bioassay-guided fractionation approach [148].

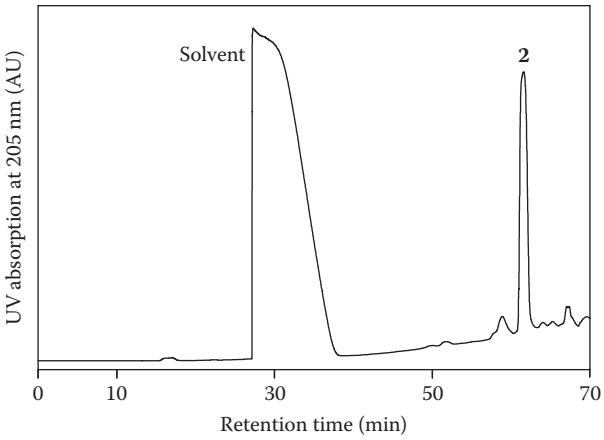


FIGURE 33.14 Preparative reversed-phase (RP)-HPLC chromatogram showing the separation of falcarindiol (2) from minor impurities in a crude polyacetylene fraction, which originated from the separation of a carrot root extract by silica gel flash column chromatography. Solvent = ethyl acetate. Separations performed on a RP Develosil ODS-HG-5 HPLC column (RP-18, 250 × 20 mm ID, Nomura Chemical Co., Seto, Japan) at 25 ºC using the following solvent gradient: methanol–water, 0 min (20:80), 50–60 min (100:0), 70–80 min (20:80). All increases/decreases in the gradient were programmed as linear. Flow rate: 5 mL/min. Injection volume: 25 mL. Detection: photodiode array (PDA) detector operating from 200–595 nm. Acquisition off at 70 min.

33.4 ANALYTICAL HPLC

33.4.1 Survey of the Separation of Polyacetylenes by Analytical HPLC

Numerous publications have described the analysis of polyacetylenes by analytical HPLC. The majority of these deal with the use of RP C18 columns such as Econosphere C18 [198], LiChrospher 100 RP-18 [121,122,139,183,191,199], LiChrosorb RP-18 [123,139,200,201], Luna 3µ C18(2) 100A [12,202,203], Purospher STAR RP-18 [12,204], Sperisorb 5S or Develosil ODS [28,43,120], Varian RP-MCH-10 [96,99–101,103], and Zorbax Rx-C18 [19,21]. In most cases, RP-HPLC separations are performed by gradient elution using different proportions of MeOH/H_2O or MeCN/H_2O as mobile phases. Polyacetylenes are normally easy to identify in extracts by PDA detection due to their often-characteristic UV spectra (Section 33.2). The detection wavelength(s), solvent gradients, and other parameters depend on the complexity and chemical structure of the polyacetylenes, as illustrated in the following examples for the analysis of falcarinol-type polyacetylenes from carrots and alkamides and 2-ketoalkenes and -alkynes from *Echinacea* species by analytical HPLC.

33.4.1.1 Analytical HPLC Separation of Falcarinol-Type Polyacetylenes

Polyacetylenes of the falcarinol type have very characteristic UV spectra due to their conjugated triple bonds and hence, they are easily identified in extracts by UV detection [1,12,28,43]. In most cases, falcarinol-type polyacetylenes have only two conjugated unsaturated bonds in their structure (Figure 33.1), and therefore the excitation coefficients ($\varepsilon < 6000$ for two triple bonds in conjugation) of these compounds at their characteristic UV maxima are low, as is the sensitivity [1,12,28,43]. Therefore, detection at 205 nm is preferred for polyacetylenes of the falcarinol type, although their characteristic UV maxima occur above 225 nm (Figure 33.12). Detection at 205 nm improves UV sensitivity by approximately 10-fold. This is especially important for the Apiaceae plant species that have low concentrations of these polyacetylenes, as, for example, in carrots and other root vegetables where the concentration can be as little as 5 mg/kg fresh weight [19,186,194,195,202]. However, depending on the extracts investigated, many other compounds also absorb UV light at 205 nm, and therefore the HPLC profile is much more complex at detection wavelengths around 200 nm. In Figure 33.15, a typical analytical RP-HPLC chromatograms at 205 nm of EtOAc extracts

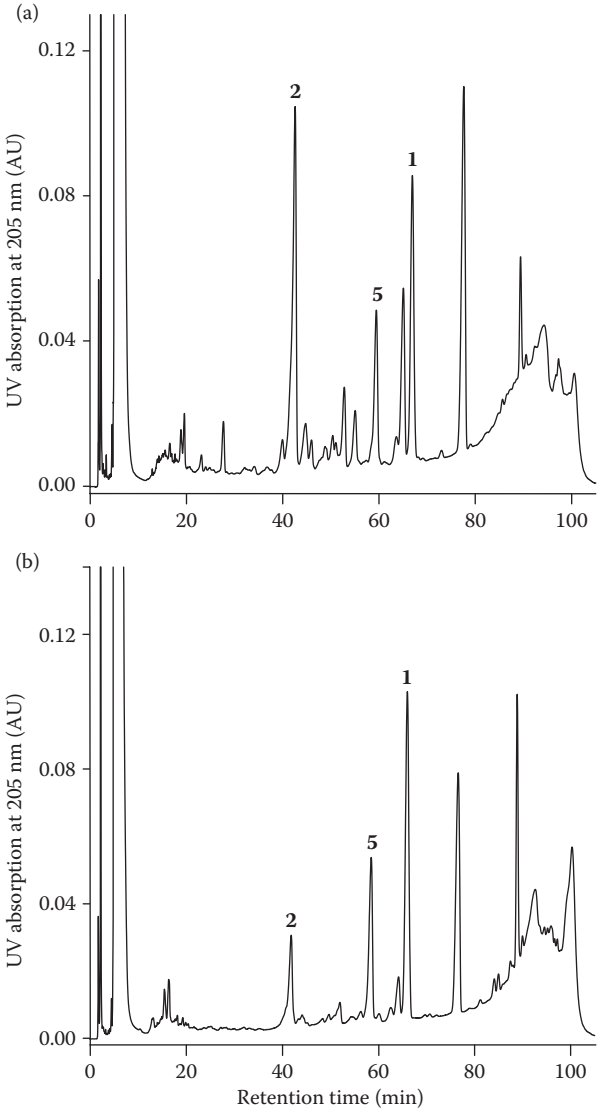


FIGURE 33.15 Analytical reversed-phase HPLC chromatograms showing the separation of the major polyacetylenes falcarinol **(1)**, falcarindiol **(2)**, and 3-*O*-acetylfalcarindiol **(5)** from ethyl acetate extracts of carrot roots from two carrot genotypes (a) 'Bolero' and (b) 'Line 2', respectively. Separations performed on a RP-Luna 3µ C18(2) 100A column (3 µm; 150 × 4.6 mm ID, Phenomenex, CA, USA) at 40° C using the following solvent gradient: methanol–water, 0–5 min (20:80), 10 min (50:50), 30 min (53:47), 45–50 min (65:35), 70–72 min (75:25), 90–95 min (95:5), 100–110 min (20:80). Flow rate: 1 mL/min. Injection volume: 20 µL. Detection: photodiode array (PDA) detector from 200–595 nm. Acquisition off at 105 min.

of carrot roots from two different carrot genotypes are shown, and as can be seen from the HPLC chromatograms, many additional peaks are observed that are not related to polyacetylenes (revealed by UV-PDA). Therefore, despite the complex RP-HPLC chromatograms of carrot root extracts at 205 nm, the polyacetylenes are separated from additional components allowing quantification of the polyacetylenes in the extracts.

In contrast, the numbers of additional peaks not related to polyacetylenes are limited in EtOAc extracts of American ginseng (*Panax quinquefolium*) root, where the concentration of polyacetylenes

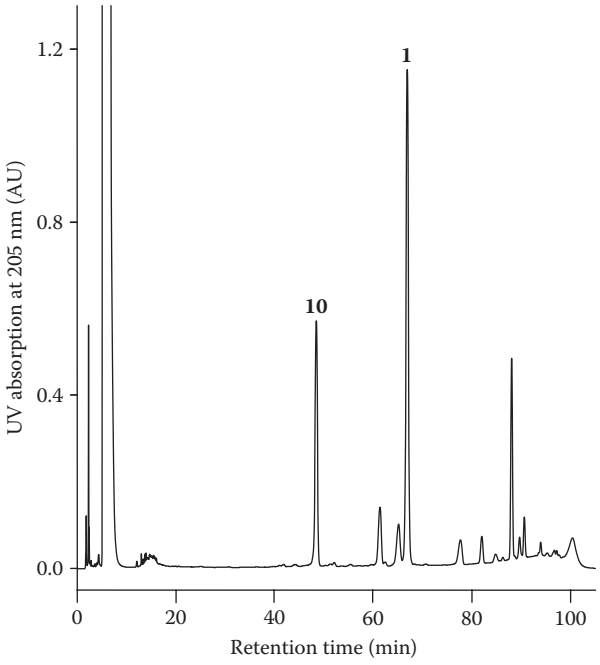


FIGURE 33.16 Analytical reversed-phase HPLC chromatogram of an ethyl acetate extract of American ginseng root showing a good separation of the two major polyacetylenes falcarinol **(1)** and panaxydol **(10)**. Separations performed on a RP-Luna 3µ C18(2) 100A column (3 µm; 150 × 4.6 mm ID, Phenomenex, CA, USA) at 40 ºC using the following solvent gradient: methanol–water, 0–5 min (20:80), 10 min (50:50), 30 min (53:47), 45–50 min (65:35), 70–72 min (75:25), 90–95 min (95:5), 100–110 min (20:80). Flow rate: 1 mL/min. Injection volume: 20 µl. Detection: photodiode array (PDA) detector from 200–595 nm. Acquisition off at 105 min.

is relatively high (between 50–2000 mg/kg fresh weight [203–205]) compared to carrots. A typical analytical RP-HPLC chromatogram at 205 nm of an EtOAc extract of the roots of American ginseng is shown in Figure 33.16, with falcarinol and panaxydol being the major polyacetylenes. Quantification of polyacetylenes by analytical RP-HPLC is performed by using an appropriate internal standard [19] or a calibration curve of authentic polyacetylene standards [186,193,202–205]. Validation of the HPLC metod used for the determination of polyacetylenes of the falcarinol type in carrot and ginseng root extracts has been performed [202–205]. It is a rather robust method with overall intraday and interday variation of lesss than 3.3% and with average recovery rates over 96% and a level of detection and quantification of falcarinol-type polyacetylenes from carrots less than 0.19 µg/mL and 0.42 µg/mL, respectively [202].

33.4.1.2 Analytical HPLC Separation of Alkamides and 2-Ketoalkenes and -alkynes from *Echinacea* Species

As described in Section 33.2, alkamides and 2-ketoalkenes and -alkynes in *Echinacea* species can to some extent be separated by NP TLC and tentatively identified based on R_f values and specific color reactions using a suitable visualization reagent. By analytical HPLC, alkamides and 2-ketoalkenes and -alkynes can be effectively separated within 40 min using a RP-18 column such as a LiChrospher 100, and a solvent system consisting of MeCN and water, as illustrated in Figures 33.17 and 33.18 for the HPLC analysis of *Echinacea pallida* and *E. purpurea* root extracts, respectively. The optimal gradient for the separation of the lipophilic constituents in extracts of *Echinacea* spp. seems to be one starting with a mobile phase consisting of MeCN–H_2O (40:60), followed by a moderate increase in solvent strength up to 35 min, and ending with a mobile phase consisting of

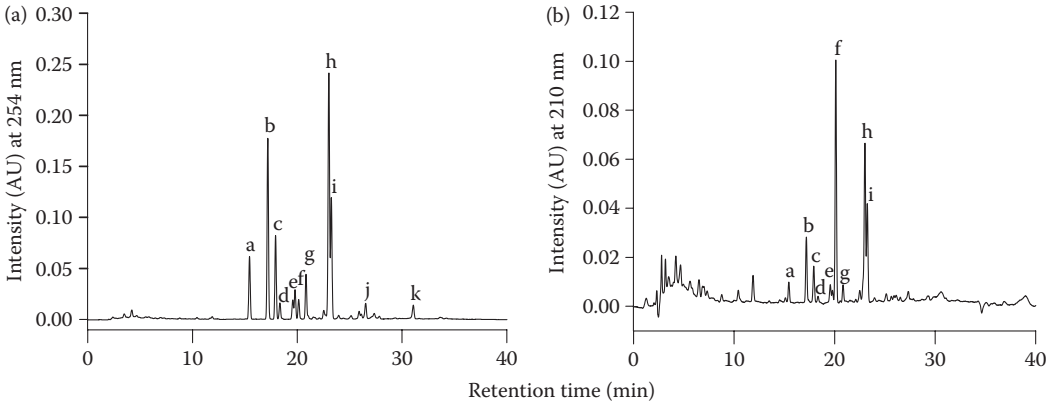


FIGURE 33.17 Separation of polyenic and polyacetylenic alkamides from an *Echinacea purpurea* root extract by analytical HPLC at detection wavelengths 254 nm (a) and 210 nm (b). Separations performed on a LiChrospher 100 RP-18 column (5 µm; 250 × 4.6 mm ID, Merck, Darmstadt, Germany) at 25 ºC using the following solvent gradient: methanol–water, 0 min (40:60), 35 min (80:20), 45 min (40:60), 50 min (40:60). Detection: photo diode array (PDA) detector from 200–400 nm. Acquisition off at 40 min. Peak identification: a = compound **137;** b = **139;** c = **138;** d = **140;** e = **155;** f = **143;** g = **141;** h = **151;** i = **152;** j = **153;** and k = **154.**

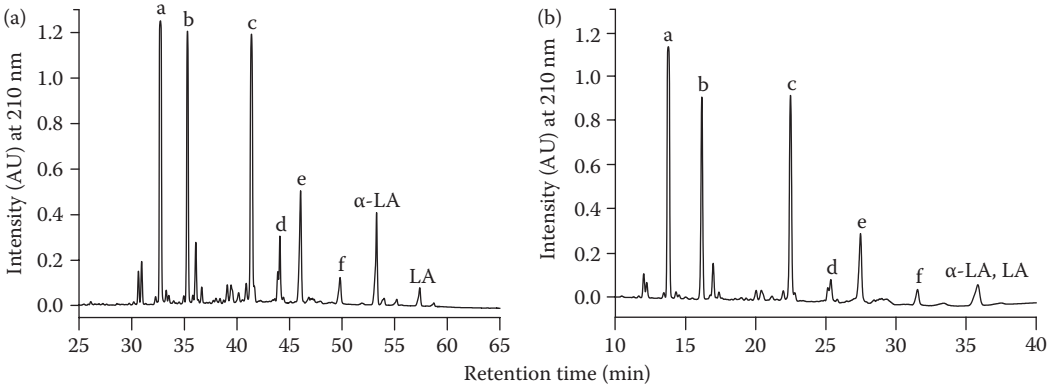


FIGURE 33.18 Separation of polyenic and polyacetylenic ketones by analytical HPLC from an *Echinacea pallida* root extract by different solvent gradients. Separations performed on a LiChrospher 100 RP-18 column (5 µm; 250 × 4.6 mm ID, Merck, Darmstadt, Germany) at 25 ºC using the following solvent gradient: (a) methanol–water, 0 min (10:90), 60 min (100:0), 70 min (100:0), 80 min (10:90), 90 min (10:90); (b) methanol–water, 0 min (40:60), 35 min (80:20), 40 min (40:60), 50 min (40:60). Detection: photodiode array (PDA) detector from 200–400 nm. Acquisition off at 65 min (a) or 40 min (b). Peak identification: a = compound **156;** b = **157;** c = **158;** d = **159;** e = **160;** and f = **161** and **162;** α-LA = α-linolenic acid; LA = linoleic acid.

80% MeCN and 20% H_2O. A much lesser increase in solvent strength starting with a mobile phase consisting of MeCN–H_2O (10:90) and ending at 100% MeCN at 60 min has a major impact on the retention time (R_t) of the lipophilic constituents. However, it has no major impact on the separation of the compounds with the exception of the highly lipophilic constituents such as the fatty acids α-linolenic acid and linoleic acid (Figure 33.18).

The elution sequence of the alkamides on the RP-18 column is predominantly influenced by the chain length (as shown by the R_t of the polyacetylenic alkamides **137/138** or **140/141**) and the number of double bonds (R_t of compounds **151**, **152**, **153**, and **154**) and triple bonds (R_t of **138** and **155**). Isomerism of double bonds (*E* or Z) has a larger effect on elution sequence of polyacetylenic alkamides (R_t of compounds **137** and **139**) compared to polyenic alkamides (R_t of compounds **151**

and **152**). The elution sequence of 2-ketoalkenes and -alkynes is clearly also influenced by chain length (R_t of compounds **156/157** and **158/159**) and the number of double and triple bonds, as shown by the comparison of the R_t of the polyacetylenic ketone **159** with that of the 2-ketoalkyne **160** and the 2-ketoalkenes **161** and **162**. Isomerism of double bonds clearly has no or only a minor effect on the elution sequence of these compounds (R_t of **161** and **162**).

From the HPLC analysis of the hexane extracts of the roots of *E. purpurea* (Figure 33.17), it can be concluded that the lipophilic constituents are alkamides that mainly possess a 2,4-dienoic acid moiety, while 2-ketoalkenes and -alkynes are the main constituents in the hexane extracts of the roots of *E. pallida* (Figure 33.18). The various structural types of lipophilic constituents are easily distinguished by their UV spectra that are recorded on-line by a PDA detector (Figures 33.10 and 33.11). Alkamides that possess a 2,4-dienoic moiety showed an absorption maximum at 259 nm (Figure 33.10), and additional conjugated olefinic or acetylenic/olefinic groups as in compounds **151**, **152**, and **155** resulted in a shoulder peak around 230 nm. Alkamides that possess a 2-monoenoic moiety had an absorption maximum at 210 nm [122,183]. Based on these observations the detection wavelengths for the analysis of alkamides in *Echinacea* spp. by HPLC-PDA are 210 nm and around 260 nm [122,183]. The 2-ketoalkenes and -alkynes of *E. pallida* showed different UV spectra with λ_{max} around 230 nm for the 2-ketoalkenes **160**–**162**. The 2-ketoalkynes showed characteristic absorption maxima above 220 nm, typical for polyacetylenes with a diyne chromophore (compounds **158** and **159**) and a diyne-ene chromophore (compounds **156** and **157**). Both the 2-ketoalkenes and -alkynes show uncharacteristic absorption maxima in the area from 200–215 nm. Therefore, the detection wavelength for the analysis of 2-ketoalkenes and -alkynes in *E. pallida* by HPLC-PDA is 210 nm, which also makes it suitable for optimal detection of alkamides.

HPLC-PDA is clearly effective for both qualitative and quantitative analysis of alkamides and other lipophilic constituents in *Echinacea* species. Quantification of the compounds are made by using an appropriate internal standard or by using calibration curves of authentic representative standards that can be purchased or easily isolated by preparative HPLC from crude fractions as described in Section 33.3.

33.4.2 LC-MS Analysis of Polyacetylenes

LC-MS methodologies have been used to characterize purified polyacetylenes and to determine the polyacetylene profile in extracts of higher plants [19,21,113,119,185]. However, obtaining a high degree of sensitivity by LC-MS requires much effort and does not seem to be suitable for characterization and quantification of small amounts of polyacetylenes in plant extracts. Therefore, HPLC-PDA and/or GC-MS are preferred for both qualitative and quantitative analysis of plant extracts of polyacetylenes [12]. For the analysis of samples containing minute amounts of polyacetylenes under the detection and quantification limit of HPLC-PDA systems, such as in biological fluid samples, the use of highly sensitive LC-MS equipment is required. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) or similar techniques are characterized by their ability to examine selectively the fragmentation of particular ions and thereby increase their sensitivity substantially. The higher selectivity and sensitivity of LC-MS/MS compared to the common LC-MS techniques makes the LC-MS/MS method an excellent tool for the examination of specific compounds appearing in very low concentrations in complex matrices such as blood or urine samples [12,206–209]. LC-MS/MS techniques have been widely applied for the determination of bioavailability of carotenoids [207] and citrus limonoids [208] in human plasma samples and phytoestrogens in milk samples [209] but also to detect and quantify polyacetylenes of the falcarinol type and alkamides in biological fluid samples [12,206].

The bioavailability of falcarinol (**1**) and falcarindiol (**2**) in humans has been studied. When falcarinol and falcarindiol were administered orally via carrot juice, they were rapidly absorbed, reaching maximum concentrations in serum of approximately 3 ng/mL at 2 h after dosing [12,210–212]. The detection and quantification of falcarinol and falcarindiol in plasma samples was performed by LC combined with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS).

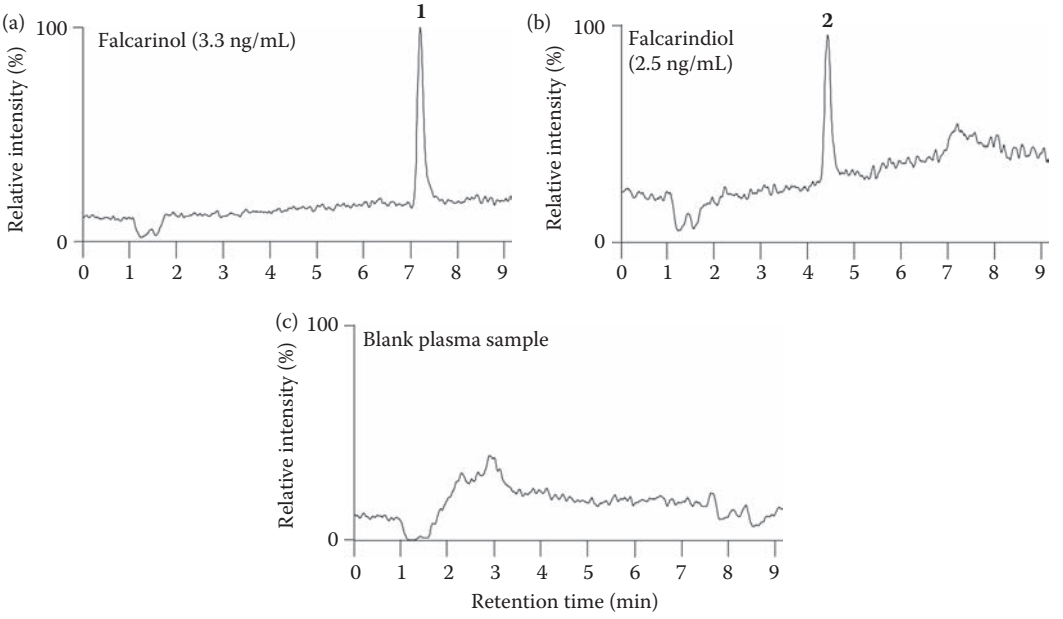


FIGURE 33.19 Liquid chromatography–mass spectrometry (LC-MS) analysis of falcarinol and falcarindiol in plasma samples. Multiple reaction monitoring (MRM) chromatograms obtained for (a) falcarinol (**1**) in plasma (concentration 3.3 ng/mL), (b) falcarindiol (**2**) in plasma (concentration 2.5 ng/mL), and (c) blank plasma sample (control). Positive electrospray ionization MS data were obtained by LC-MS/MS on a Quattro LC (Micromass) using N_2 as desolvation gas at a flow rate of 650 L/hr and a temperature of 400 °C. Potentials applied on the electrospray capillary and on the cone were 3.5 kV and 20 V, respectively. Detection: MRM method using argon as collision gas and the mass transition *m/z* 227→91 (falcarinol) and *m/z* 247→91 (falcarindiol). Separations were performed on a Chromolith Performance RP-18e column (100 × 4.6 mm ID, Merck, Darmstadt, Germany) using a binary gradient consisting of 0.1% formic acid in water and methanol. Flow rate: 0.3 mL/min. Extraction recoveries were in the range 79–100% depending on the analyte concentration.

The polyacetylenes were extracted from plasma samples with MeCN and analyzed directly by LC-ESI-MS/MS using falcarinol and falcarindiol as external standards. Fragment ions of falcarinol and falcarindiol were generated in the electrospray positive-ion mode (ESI+) with argon as collision gas using the mass transitions *m/z* 247 $[M - H_2O + Na]^+ \rightarrow 91$ and *m/z* 227 $[M - H_2O + H]^+ \rightarrow 91$ for detection of falcarindiol and falcarinol, respectively [12,210–212]. The number of ions produced permitted the development of an efficient analytical method based on multiple reaction monitoring (MRM) acquisition mode, as illustrated in Figure 33.19. The mass traces from the MRM method clearly demonstrate its selectivity. The quantification limits for both polyacetylenes were around 0.5 ng/mL [12,210–212], illustrating the sensitivity of the method and hence, its usefulness for the determination of polyacetylenes of the falcarinol type in plasma samples and other samples with low concentrations of these compounds.

Alkamides are suspected to contribute to the pharmacological effects of *Echinacea* preparations as described in Section 33.1.3. A sensitive and specific method has for example been developed for the identification and quantification of alkamides such as compounds **138** and **151–154** in human plasma by LC-ESI-ion trap-MS/MS after administration of *Echinacea* preparations [206,213]. The alkamides gave protonated precursor molecular ions $[M + H]^+$ in the MS mode and produced intense product ions in the MS/MS spectra. The mass spectra from the fragmentation produced in the MS/MS mode were acquired in the selected reaction monitoring (SRM) scan mode resulting in an LC-MS/MS method with high selectivity and sensitivity suitable for quantification of alkamides in plasma samples and for pharmacokinetic studies. For example, the polyacetylenic alkamide **138**

gave a precursor molecular ion at *m/z* 244 $[M + H]^+$ and an intense fragmentation ion at *m/z* 145. The maximum concentration of the polyacetylenic alkamide **138** was reached within minutes (1.54 ng/mL plasma) after oral administration of a 60% EtOH extract of *Echinacea angustifolia* roots in human volunteers. In total the bioavailability of six alkamides was determined, with maximum concentrations from 0.00 to 10.88 ng/mL reached within minutes [206].

33.5 CONCLUSION

Polyacetylenes constitute a class of highly unsaturated secondary metabolites with a relatively limited distribution in higher plants, being present mainly in the Apiaceae, the Araliaceae, and the Asteraceae plant families. Polyacetylenes have shown various significant pharmacological activities that to some extent relate to their reactivity toward biomolecules. In addition, the lipophilic character of polyacetylenes makes these compounds suitable candidates for crossing the lipophilic cell membranes and hence being bioavailable in vivo. Consequently, polyacetylenes have the potential to exert their pharmacological activities in vivo, and therefore it is likely that some polyacetylenes play an important role in the health promoting properties of certain medicinal and food plants. Furthermore, the significant pharmacological activities of some polyacetylenes indicate their potential in the development of effective herbal medicines and drugs for the prevention and treatment of various diseases. Hence, it is of utmost importance to have good and reliable analytical methods for the qualitative and quantitative analysis of these compounds. The present review has demonstrated that HPLC in combination with UV and MS detection is an excellent analytical method for the analysis of these compounds in different matrices such as extracts and biofluids. Isolation of polyacetylenes in large amounts, such as for clinical and preclinical trials, can be done rather effectively by the use of semi- and/or preparative HPLC combined with other chromatographic techniques, as has also been demonstrated in this review.

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34 HPLC of Quinonoid Phytochemicals

Subhalakshmi Ghosh, Madhushree Das Sarma, and Banasri Hazra

CONTENTS

34.1 INTRODUCTION

Plants and plant-derived products have continually been integral partners in the progress of human civilization since time immemorial, particularly in association with the customary health-care practices of the hoi polloi. Green plants produce and store an enormous variety of unique phytochemicals as metabolites. Many of these chemical entities serve as vital constituents in our foods, spices, and beverages, while some of them are extractable as chemical feed stocks for medicinal and nutraceutical preparations, as well as for synthesis of modern drugs. Development of efficient techniques for

detection and separation, followed by recent findings on the significant pharmacological properties of these molecules, could provide a scientific rationale for some time-honored ethnobotanical practices. Therefore, in recent times, advanced analytical procedures for critical evaluation of plant-derived compounds are gaining fresh ground on account of the massive surge in consumption of herbal prescriptions taking place all over the world.

In this context, several bioactive quinonoid phytochemicals and their analogues have been found to serve as major "marker" constituents of the medicinal plants frequently used in commercially important herbal formulations prescribed in Ayurveda, Siddha, Unani, Pen Ts'ao, Kampo, and numerous other traditional systems prevailing in and around Asia. Actually, quinones are ubiquitous components of the respiratory mechanism, serving as vital links in the electron transport chain by virtue of facile redox interactions in the metabolic pathway of living cells [1]. Furthermore, the unique structural feature of these compounds also contributes to their inherent cytotoxicity, so that the host plants might use it as a defense mechanism against invading bacteria, fungi, or parasites [2].

In this article, several quinonoids recognized for biological activity will be presented with reference to their detection, analysis, and separation from plant sources by the application of high performance liquid chromatography (HPLC). Again, plant-derived anthraquinonoids occurring as chromatic pigments in plants are notable for their characteristic bright colors, hence are used as dyestuffs in paintings and textiles, while a few naphthoquinonoids have also been utilized as colorants in cosmetics, nutraceuticals, and the food-processing industries. Interestingly, HPLC analysis of natural dyes has facilitated investigation of samples from ancient civilizations collected for archaeological studies. In fact, the development of analytical techniques pertaining to the detection/analysis of miniscule amounts of analytes was typically motivated by the specific implication of these phytochemicals, particularly in relation to pharmacological and medicinal aspects. Thus, recent advances in HPLC column specification and composition of the stationary phase, along with tandem mass spectrometry (MS/MS), and hyphenated photodiode array (PDA), fluorimetric, and electrochemical detection techniques will be pointed out with regard to the analysis of 1,4-quinonoid constituents of terrestrial and cultured plant samples, fruits/vegetables and algae, and dyes and colorants, as well as biological tissues. Actually, a large number of publications in the new millennium have reported the variations or modifications introduced in the aforesaid conditions for the particular analyte/biological sample, which we have attempted to compile in Table 34.1 in the sequence of their structural order, that is, benzo-, naphtho-, and anthraquinonoids. However, numerous documents typically aiming at standardization of the commercial formulations of traditional Chinese medicines (TCM) with heterogeneous character, have not been included here, by and large, barring a few examples dealing with specific herbal composition.

34.2 BENZOQUINONES

34.2.1 Thymoquinone

Thymoquinone, a benzoquinonoid (Figure 34.1), was identified by El-Dakhakhny [3] as the major component of the volatile oil extract of *Nigella sativa* Linn. (Rananculaceae) seeds, called "black cumin." The herb is widely grown in different parts of the world, and its seeds are used as a spice and condiment. Traditionally, the "black seed," known as "Kalonji" in the Indian subcontinent and "Habat-ul-Sauda" in Arabic, was believed to be a panacea for all ailments, hence recommended for a wide range of diseases, including asthma, chronic headache, migraine, chest congestion, dysmenorrhea, obesity, paralysis, rheumatism, hypertension, flatulence, diarrhea, and so on, while the seed oil was used as a carminative, diuretic, lactagogue, and vermifuge [4].

Pharmacological studies recently conducted on thymoquinone found it to be a potent anti-inflammatory [5] and anti-arthritic [6] compound, broadly justifying the traditional therapeutic uses of *N. sativa* seeds. Relevant mechanisms of action through inhibition of cyclooxygenase and

TABLE 34.1
Analysis of Plant-Derived Quinonoids by HPLC Techniques

Sample for Analysis	Detection Technique
Thymoquinone	
Nigella sativa (seed) [24,25,170]	LC-UV [24,25,170]
Plumbagin	
Plumbago zeylanica (root) [60,63]; *P. zeylanica* (root and stem) [58]; *P. zeylanica* (root, stem, and leaf) [171]; *P. europea* (root) [172]; *P. rosea* (root, cell culture) [173]; *Diospyros usambarensis* (root bark) [59]; *Drosera binata* (cell culture) [39]; *D. rotundifolia* [31]; *Dionaea muscipula* [31]; *Impatiens glandulifera* [31]; *Paulownia tomentosa* [31]; plasma of rats fed with plumbagin [64]	LC-UV [39,58–62,171–174]; LC-PDA [31]; LC-MS [63,64]
Juglone	
Juglans regia (leaf) [68]; *J. regia* (leaf and peel) [175]; *J. regia* (shoots) [69]; *J. regia* [31]; *J. nigra* (root) [176]; extracts of textile fibers [177]	LC-UV [61,62,68,175,176]; LC-PDA [31,69,177]
Lawsone	
Impatiens glandulifera (stems, leaves, flowers) [178]; *I. glandulifera* [31]; *I. capensis* [178]; *I. noli-tangere* [178]; *I. parviflora* [178]; *Lawsonia inermis* (root culture) [179]; *L. inermis* (leaf) [180]; hair dye [81]; extracts of textile fibers [168,177]	LC-UV [61,62,178,179]; LC-PDA [31,81,168,177,180]
Lapachol	
Tabebuia avellanedae (bark) [94]; *T. avellanedae* (heartwood) [95]; extracts of textile fibers [177]	LC-UV [94,95,181–183]; LC-PDA [177]
Alkannin and Shikonin	
Lithospermum eryhthrorhizon (root/callus culture) [123,124,126,189,190,196]; *M. cephalotes* [60]; *M. euchroma* (root) [186,187]; *Arnebia euchroma* [123]; *A. guttata* [123]; *Alkanna tinctoria* [124]; *A. densiflora* (roots/ callus) [188]; *Alkanna tinctoria* (root) [128]; *A. cappadocica*, *Echium italicum*, *E. russicum*, *E. vulgare*, *Onosma sericeum*, *O. microcarpum*, *Anchusa leptophylla*, *A. undulate*, and *Arnebia* sp. (root) [125]; *Arnebiae* (radix) and commercial preparation (Tzyy-Yun-Gau) [123]; "Shiunko" ointment containing *L. erythrorhizon* roots [123]; alkannin, shikonin, shikalkin (commercial samples), *Alkanna* spp. (root), pure mixture of isohexenylnaphthazarins pigments, microcapsules containing alkannin and shikonin inclusion complexes [127]; *Arnebia euchroma*, *A. guttata*, *L. erythrorhizon*, *Onosma paniculatum*, *O. exsertum*, *O. confertum*, *O. hookerii* var. *longiflorum*, *O. hookerii*, *O. waltonii* [129]; *Alkanna calliensis*, *A. corcyrensis*, *A. graeca*, *A. methanaea*, *A. orientalis*, *A. pindicola*, *A. primuliflora*, *A. sieberi*, *A. stribrnyi*, *A. tinctoria* (root) [191]; *Boraginaceous* spp. produced (root culture) [192]; *Alkanna tubulosa*, *A. mughlae*, *A. tinctoria* (root) [193]; *Onosma echioides* (roots) [194]; *Arnebia* sp., including *A. euchroma* (cultures) [195]	LC-UV [123,125,184,185,187–189,193,196]; Chiral LC-UV [124–128]; LC-PDA [129,186,190,194]; LC-PDA-MS [191,192]; rapid preparative-scale HPLC [195]

(*Continued*)

TABLE 34.1 (CONTINUED)
Analysis of Plant-Derived Quinonoids by HPLC Techniques

Sample for Analysis	Detection Technique
Phylloquinone	
Sunflower oil, corn oil, olive oil, pumpkin seed oil, potato, carrot, cauliflower, broccoli [103], oatmeal, milk, yogurt, Emmentaler cheese, eggs, human serum [100], trout liver [103]; serum of human volunteer fed with broccoli [197]; plasma of human osteoporotic patients [198]; blood sample of healthy human volunteer and from patients with hyperlipidemia, and elective knee arthroplasty [99]; red alga (*Porphyridium purpureum*, *Cyanidium caldarium*) [106]; raw spinach leaves and brussels sprouts [199]; intravenous fat emulsions and soybean oil [200]; rat liver [101]; olive oil, chard, and human plasma [201]; food samples of human diet [104]; kale (*Brassica oleracea* var. *Acephala*) [202]; cultured human cell lines (HepG2 and MG-63) [203]	LC-UV [103,104,106,197,200,201]; LC-FL [99,198,199]; LC (PO-CL)-UV [100]; LC-ECD-FL [101]; LC-APCI-MS [202,203]
Diospyrin	
Diospyros montana (stem bark) [62]; *Euclea crispa* (root) [140]; *E. divinorum* (root) [140]; *E. natalensis* (root) [140]; *E. pseudebenus* (root) [140]; *E. undulate* (root) [140]	LC-UV [62,140]
Aloe emodin	
Aloe barbadensis (leaf) [204]; *A. natalensis* (leaf) [204]; *Cassia alata* (root) [151]; *Polygonum cuspidatum* (rhizome) [152]; *Rheum officinale* (root) [205]; rhubarb [149,150,206,207]; *Senna angustifolia* (leaf, fruit) [208]; *S. acutifolia* (leaf, fruit) [208]; Dachengqi Tang (Chinese purgative) [209]; plasma of mouse treated (i.p.) with aloe emodin [210]; plasma, urine, and CSF of rats fed with rhubarb [211]; plasma of rats fed with Da-Cheng-Qi decoction and Xiao-Cheng-Qi decoction [153,212]; plasma of rats fed with Xiexin decoction [213]	LC-UV [151,204,205,207,209,212]; LC-PDA [149,150,152,206,208,211]; LC-MS [151–153]; LC-FL [210,213]
Emodin	
Cassia alata (root) [151]; *Polygonum cuspidatum* (rhizome) [152,214]; *P. multiflorum* (culture) [215]; *Rheum emodi* (rhizome) [148]; *R. officinale* (root) [205]; rhubarb [149,150,206,207,217]; *Senna* (leaf) [217]; *Senna angustifolia* (leaf, fruit) [208]; *S. acutifolia* (leaf, fruit) [208]; Dachengqi Tang (Chinese purgative) [209]; Danning tablet [218]; "YIGONG" capsule [219]; plasma, urine, and CSF of rats fed with rhubarb [211]; plasma of rats fed with Da-Cheng-Qi and Xiao-Cheng-Qi decoction [153]; plasma of rats fed with Xiexin decoction [213]; extracts of textile fibers [177]	LC-UV [151,205,207,209,215,217,218]; LC-PDA [148–150,152,177,206,208,211,214,219]; LC-MS [151–153,218]; LC-FL [213]
Rhein	
Cassia alata (root) [151]; *C. senna* var. *angustifolia* (pods) [220]; *Rheum officinale* (root) [205]; *R. palmatum* (root) [211]; *R. undulatum* (root) [216]; rhubarb [149,150,206,207,217]; Senna (leaf) [217]; *Senna angustifolia* (leaf, fruit) [208]; *S. acutifolia* (leaf, fruit) [208]; Dachengqi Tang (Chinese purgative) [209]; plasma of rats fed with Da-Cheng-Qi decoction and Xiao-Cheng-Qi decoction [153,212]; plasma, urine, and CSF of rats fed with rhubarb [211]; plasma of rats fed with Xiexin decoction [213]	LC-UV [151,205,207,209,212,216,217]; LC-PDA [149,150,206,208,211,220]; LC-MS [151,153,216,220]; LC-FL [213]

Chrysophanol	
Cassia alata (root) [151]; *Rheum emodi* (rhizomes) [148]; *R. officinale* (root) [205]; rhubarb [149,150,206,207,217]; Senna (leaf) [217]; *Senna angustifolia* (leaf, fruit) [208]; *S. acutifolia* (leaf, fruit) [208]; Dachengqi Tang (Chinese purgative) [209]; Danning tablet [218]; plasma, urine, and CSF of rats fed with rhubarb [211]; plasma of rats fed with Da-Cheng-Qi decoction and Xiao-Cheng-Qi decoction [212]; plasma of rats fed with Xiexin decoction [213]	LC-UV [151,205,207,209,212,217,218]; LC-PDA [148–150,206,208,211]; LC-MS [151,218]; LC-FL [213]
Physcion	
Cassia alata (root) [151]; *Polygonum cuspidatum* (rhizome) [152]; *P. multiflorum* (culture) [215]; *Rheum emodi* (rhizomes) [148]; *R. officinale* (root) [205]; rhubarb [149,206,207]; *Senna angustifolia* (leaf, fruit) [208]; *S. acutifolia* (leaf, fruit) [208]; Danning tablet [218]; plasma, urine, and CSF of rats fed with rhubarb [211]; plasma of rats fed with Xiexin decoction [213]	LC-UV [151,205,207,215,218]; LC-PDA [148,149,152,206,208,211]; LC-MS [151,152,218]; LC-FL [213]
Alizarin	
Rubia tinctorum (root) [164–167,221–224]; *Rubia tinctorum* (culture) [162–164,225]; *Rubia peregrina* (culture) [163]; wool dyed with madder [154,226]; extracts of textile fibers [168,169,177]; extracts of dyes from historical maps [228]; extracts from archaeological samples [229]; natural red dyestuff [227,230]	LC-UV [162–164,167,169,221–223,231]; LC-PDA [154,163,165,166,168,177,224–228]; LC-MS [154,166,169,224,227,229,230]
Purpurin	
Rubia tinctorum (root) [164–167,221,222,224]; *Rubia tinctorum* (culture) [164,225]; wool dyed with madder [154,226]; extracts of textile fibers [168,169]; extracts of dyes from historical maps [228]; extracts from archaeological samples [229]; natural red dyestuff [227,230]	LC-UV [164,167,169,221,222,231]; LC-PDA [154,165,166,168,177,224–228]; LC-MS [154,166,169,224,227,229,230]
Lucidin	
Rubia tinctorum (root) [164–166,221,224]; *Rubia tinctorum* (culture) [164]; natural red dyestuff [230]	LC-UV [164,221]; LC-PDA [165,166,224,230]; LC-MS [166,224,230]
Quinizarin	
Rubia tinctorum (root) [165,166,224]; wool dyed with madder [154]; extracts from archaeological samples [229]	LC-PDA [154,165,166,224,229]; LC-MS [154,166,224,229]
Munjistin	
Rubia tinctorum (root) [165,166,224]; wool dyed with madder [154]; extract of textile fibers [168,177]; natural red dyestuff [227]	LC-PDA [154,165,166,168,177,224,227]; LC-MS [154,166,177,224,227]

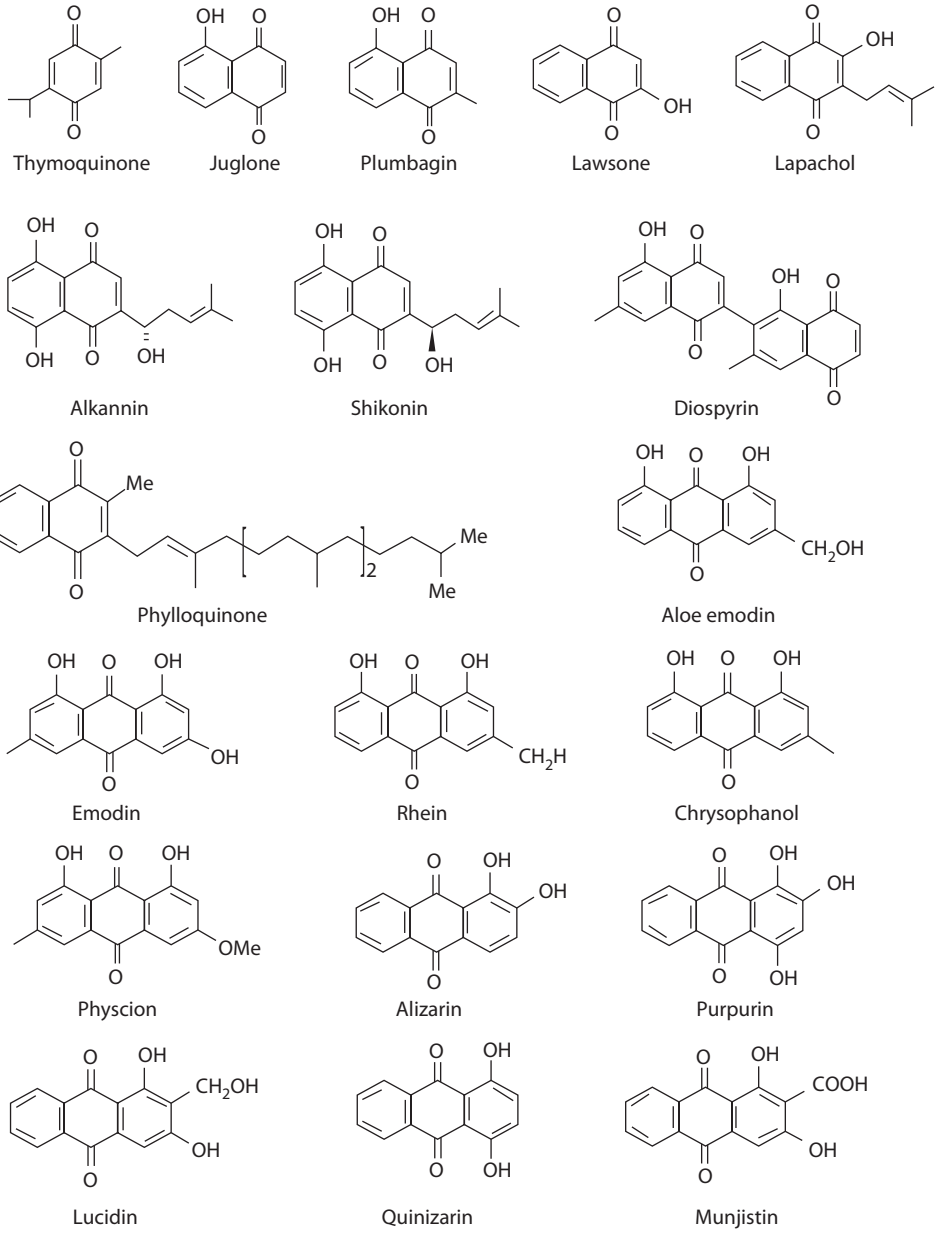

FIGURE 34.1 Structures of quinonoid phytochemicals.

5-lipoxygenase pathways in arachidonic acid metabolism [7–9], and suppression of iNOS protein [10], have been proposed recently. Thymoquinone was shown to exhibit potent hepatoprotective [11] and gastroprotective [12] properties via an antioxidant mechanism [13–15]. Significant antidiabetic activity was reported in mammalian models tested in vivo [16]. Protective efficacy of thymoquinone against cardiotoxicity [17], acute renal toxicity [18], hippocampal neurodegeneration [19], and hyperhomocysteinemia [20] in rodent models was also reported. Cytotoxicity of thymoquinone against several human cancer cell lines has also been observed, with induction of apoptotic cell death [21–23].

34.2.1.1 HPLC Determination of Thymoquinone

HPLC determination of thymoquinone content in black seed oil was performed by using an Econosphere CN column, with an isocratic mobile phase consisting of hexane:isopropanol (99:1, v/v), monitored by UV detection at 295 nm [24]. This simple method reportedly exhibited sensitivity to a lower limit of 5 nM. Subsequently, a reversed-phase liquid chromatography (RPLC) method was established [25] with a μBondapak C_{18} analytical column (Waters, MA, USA; 10 μm; 300 × 3.9 mm i.d.), using the isocratic mobile phase of water:methanol:2-propanol (50:45:5, v/v) at a flow rate of 2 mL min^{-1}, and UV detection at 254 nm. In addition to thymoquinone, three other constituents of black seed oil, viz., dithymoquinone, thymohydroquinone (detected at 294 nm), and thymol, were also separated and quantified with good resolution by this method, which was found to be sensitive and reproducible. Appropriate precautions were adopted to ensure stability of the products since the storage conditions would make a difference in the amounts of the light- and heat-sensitive constituents of the seed oil of *N. sativa*.

34.3 NAPHTHOQUINONES

Naphthoquinones, such as plumbagin, juglone, lawsone, lapachol, shikonin, and alkannin, which are presented in the following, occur as secondary metabolites in a number of plant families (*Plumbaginaceae*, *Juglandaceae*, *Boraginaceae*, *Ebenaceae*, *Iridaceae*, *Verbenaceae*, *Ancistrocladaceae*,*Avicenniaceae*,*Balsaminceae*,*Bignoniaceae*,*Droseraceae*,*Drosophyllaceae*, *Dioncophyllaceae*,*Gentianaceae*,*Nepenthaceae*,*Lythraceae*,*Scrophulariaceae*,*Euphorbiaceae*, etc.), as well as in lichens, fungi, and microorganisms.

34.3.1 Plumbagin

Plumbagin (Figure 34.1) is a hydroxynaphthoquinonoid pigment, mostly derived from roots of *Plumbago zeylanica* Linn. and other members of the Plumbaginaceae family [26]. The whole plant and root of *P. zeylanica* are well known in folk medicine for the treatment of rheumatic pain, dysmenorrhea, carbuncles, contusion of the extremities, ulcers, and elimination of intestinal parasites. Included in a number of traditional Ayurvedic formulations in India, *P. zeylanica* is also utilized in Nigerian folk medicine to treat parasitic diseases, scabies, and ulcers in Africa [27]. Again, plumbagin has also been found as a constituent of plants other than *Plumbago* spp., such as *Ancistrocladus abbreviatus* [28], *Drosophyllum lusitanicum* [29,30], *Dionaea muscipula* [31], *Diospyros* spp. [32–37], *Drosera* spp. [38,39], *Juglans regia* [40], *Nepenthes* spp. [41,42], and so on.

The biological activities of plumbagin have been studied exhaustively. Thus, experiments were carried out to evaluate the antiparasitic activity of plumbagin against *Plasmodium* and *Leishmania* spp. [41,43–47]. Reports are available on the anticancer activity of plumbagin against hepatoma, leukeamia, melanoma, and Ehrlich ascites carcinoma models induced in experimental rodent systems [46,48–50]. Recently, studies were also undertaken on human cell lines of melanoma, cervical, breast, prostate, and non-small cell lung cancers to document the significant cytotoxicity of plumbagin, in association with its potential to induce programmed cell death. The mechanistic study in this regard revealed the inactivation of NF-kappaB and Bcl-2 by plumbagin in the tested cancer cells, which involved generation of reactive oxygen species, inhibition of topoisomerase II, and modulation of p53 via c-Jun NH_2-terminal kinase-pathway [51–54]. The radiosensitizing efficacy of plumbagin was also established through studies on experimental mouse and human cancer cell lines [55,56]. In addition, there are reports on the antitubercular, anti-*Helicobactor pylori*, antibacterial, and antifungal activities of this naphthoquinonoid plant constituent [35,36,57,58].

34.3.1.1 HPLC Analysis of Plumbagin and Its Analogous Quinonoids

HPLC analysis of plumbagin and its analogous quinonoids in African plants of *Diospyros* spp. was first reported in 1984 by Marston and Hostettmann [59]. A μBondapak CN column was used with an isocratic mobile phase of *n*-hexane with 1% acetic acid and UV detection at 254 nm to separate plumbagin, juglone, 7-methyljuglone, and lawsone. Thereafter, Gupta et al. used a μSpherogel column and a mobile phase composition of *n*-hexane:chloroform:2-propanol (30:70:2, v/v/v) for analysis of *Plumbago zeylanica* roots [60]. However, none of these normal-phase HPLC methods could achieve quantitative separation of the naphthoquinonoid quinonoid (plumbagin) from another associated constituent, namely, 2, 2-dimethyl-5-hydroxy-6-acetylchromene. Therefore, subsequent workers used RPLC for detection and standardization of plumbagin and its analogous quinones in plant samples [61,62]. Recently, Wang and Huang [58] analysed 13 samples of *P. zeylanica* root and stem from different sources, using a LiChrospher 100RP18e column (5 μm; 250 × 4.0 mm i.d.; Merck), and a mobile phase composed of water:methanol with gradient elution. In this method, the limit of detecton (LOD) and limit of quantitation (LOQ) were validated to be 0.02 and 0.06 μg/mL, respectively, with good precision and accuracy. The plumabagin content, detected by UV at 254 nm, was found to be higher in the roots than in the stems of *P. zeylanica*.

At the same time, another group of workers [63] standardized a liquid chromatography system (Waters 2690) with a PDA coupled with tandem mass spectrometry (LC-MS/MS) using a ZORBAX Extend-C_{18} column (5 μm; 150 × 4.6 mm i.d.), which was eluted with a mobile phase of water:methanol (10:90, v/v). Multiple reaction monitoring was used to monitor the transition of the deprotonated molecule *m/z* 187 [M-H]- to the product ion *m/z* 159 [M-H-CO]- for plumbagin analysis. The method demonstrated accurate and reproducible separation of plumbagin from herbal extracts, and the LOD and LOQ were determined to be 0.5 and 1 ng/mL, respectively, with the mobile phase of water–acetonitrile (40:60, v/v) at a flow rate of 0.8 mL/min. It was interesting to find that the method has further been validated with water:acetonitrile (40:60, v/v) used as the mobile phase at a flow rate of 0.8 mL/min to study the pharmacokinetics of plumbagin in rats. In fact, the authors have standardized this method for automated blood sampling in conscious and freely moving Sprague–Dawley rats in order to collect oral bioavailability data for preclinical research on plumbagin [64].

Recently, Babula et al. [31] reported the determination of several naphthoquinones in tissue samples of a series of plants known to possess high amounts of quinonoids, including a carnivorous species, namely *Dionaea muscipula*, and others, like *Drosera rotundifolia*, *Juglans regia*, *Impatience glandulifera*, and *Paulownia tomentosa*, in order to correlate secondary metabolite content with variation in environmental factors, like pH of the cultivation medium. The authors reportedly used liquid chromatography coupled with diode array detection (HPLC-PDA). Prior to the analysis, the plant samples were processed by performing accelerated solvent extraction (ASE) in a pressurized solvent extractor (Applied Separations). A reversed-phase Zorbax C_{18}–AAA column (3.5 μm; 150 × 4.6 mm i.d.; Agilent Technologies) was used in an isocratic elution mode with aqueous acetic acid and methanol. Thus, several monomeric naphthoquinonoid derivatives, including plumbagin, juglone, and lawsone, could be separated as major secondary metabolites from the respective host plants. In this method, the LOD and LOQ for analysis of plumbagin were found as 27 and 88.5 ng/mL, respectively.

34.3.2 Juglone

Juglone (Figure 34.1), a close analogue of plumbagin, occurs in the roots, leaves, and stems of *Juglans* spp. plants of Juglandaceae family [65], particularly in walnut hulls. Gardeners have long known that landscaping underneath or near black walnut trees could be difficult. This is because juglone is an allelopathic compound, and it exerts its effect by inhibiting certain enzymes needed for metabolic function in plants growing in the vicinity of walnut trees. In fact, it is occasionally

used as a herbicide. Juglone isolated from walnut hulls has been traditionally used as a natural dye for fabrics, particularly wool, and also as ink. Because of its tendency to create dark orange–brown stains, juglone has been used as a coloring agent for foods and cosmetics.

The biological activity of juglone, believed to be rather cytotoxic, has been studied against bacterial cultures [66] and also in human keratinocyte (HaCat) cells [40], particularly in view of its application as a natural dye. A recent report showed a potential anti-*Helicobactor pylori* effect of juglone through inhibition of three key enzymes: cystathionine gamma-synthase, malonyl-CoA:acyl carrier protein transacylase, and beta-hydroxyacyl-ACP dehydratase of bacterial metabolic system [67].

34.3.2.1 HPLC Method for Determination of Juglone

An HPLC method for determination of juglone in fresh walnut tree leaves (*Juglans regia*) was described by Gîrzu et al. [68]. RPLC elution of juglone at 99.93% purity was achieved using a linear gradient program and UV detection at 420 nm (LOD 0.065 μg) with the mobile phases of (A) water:phosphoric acid (95.5:0.5, v/v) and (B) acetonitrile:water (90:10, v/v).

HPLC-PDA was used by Solar et al. [69] to analyze seasonal variations in metabolite content, including juglone and 1,4-naphthoquinone, in annual shoots of common walnut (*Juglans regia* Linn.). A Chromsep Hypersil 5 ODS column (Chrompack, Middelburg, Netherlands; 250 × 4.6 mm i.d.), protected with a Chromsep guard column, was eluted with solvent A (2% aqueous acetic acid) and solvent B (0.5% aqueous acetic acid:acetonitrile [1:1, v/v]) as the mobile phase.

A simple and efficient method was developed in our laboratory for separation of monomeric naphthoquinone derivatives, including plumbagin and juglone, using isocratic elution with acetonitrile:water (40:60) on a μBondapak C_{18} RP column, followed by UV detection at 255 nm [62].

34.3.3 Lawsone

Lawsone (Figure 34.1), another close analogue of plumbagin and juglone, is a red–orange dye chiefly found in the leaves of the henna plant (*Lawsonia inermis* Linn., Lythraceae). Henna extracts containing 1–2% lawsone have been used as hair and skin pigments for more than 5000 years, particularly in India and the Middle East. Even today, the traditional art of henna painting (Mehndi), a form of personal decoration during marriages and festivals, uses dried henna leaves ground to a fine powder and mixed with water or oil to create a reddish–brown paste. It is applied to the skin and allowed to dry, and subsequently the paste is brushed away, leaving a reddish-brown design.

Lawsone, one of the best wool-coloring agents [70], was found to react with the keratin protein in wool, hair, and skin via Michael addition, resulting in a strong permanent stain that lasts for several weeks. The application of lawsone as a color additive was approved by the Food and Drug Administration (FDA) under 21 CFR 73.2190 for dyeing hair. However, in 2002, the Scientific Committee for Consumer Products (SCCP) evaluated the safety of lawsone as a coloring agent in hair dye products of the European Union (EU) and concluded that lawsone was mutagenic [71]. A recent study proposed the involvement of reactive oxygen radicals [72] such that this naphthoquinonoid compound could even induce severe hemolytic anemia [73]. However, no genotoxic effect was found to be associated with the application of lawsone so far [74].

The biological activity of lawsone was first documented when Tripathi et al. [75] observed the antifungal activity of this naphthoquinonoid. This was followed by discovery of its inhibitory activity on the hematoporphyrin-sensitized photohemolysis of normal and sickle cell erythrocytes [76] and suppression of the protein glycation process [77]. Isolated studies indicated its molluskicidal [78], anti-inflammatory, analgesic, and antipyretic [79] properties. Recently, lawsone was investigated as a novel class of forensic reagent for fingerprint detection. The compound was found to react with hidden fingermarks on paper surfaces to yield purple-brown impressions of ridge details that could be analyzed by photoluminescence [80].

34.3.3.1 HPLC Analysis for Detection of Lawsone

HPLC analysis for detection of lawsone as an ingredient of hair dyes was standardized with gradient elution, employing phosphate buffer:acetonitrile as the mobile phase and PDA detection in the wavelength range of 220–400 nm [81]. Babula et al. [31] also optimized the simultaneous analysis of lawsone, juglone, and plumbagin from plant samples by HPLC-PDA, with the LOD and LOQ for detection of lawsone being 28 and 94 ng/mL, respectively. A simple and efficient method was developed for separation of monomeric naphthoquinone derivatives, including plumbagin, juglone, and lawsone, using isocratic elution with acetonitrile:water (40:60) on a μBondapak C_{18} RP column, followed by UV detection at 255 nm [2].

34.3.4 Lapachol

Lapachol (Figure 34.1), another monomeric naphthoquinonoid, was isolated from the bark of the genus *Tabebuia*, especially *Tabebuia avellanedae* (Bignoniaceae). The preparation from the inner bark of this plant, known in Brazil by the names "Lapacho," "Pao d' arco," "Ipê," and so on, was used in Chagas disease and also for cancer therapy in traditional cultures of South America and the United States.

A number of preclinical studies were reported on the antiviral [82], anti-inflammatory [83], antibacterial [84], and molluskicidal [85] activities of lapachol. Substantial work was carried out on the antiparasitic property of lapachol, which was found to be an effective inhibitor of *Trypanosoma cruzi* [85,86], *Leishmania braziliensis* [87], *L. amazonensis* [88], and *Plasmodium falciparum* [89]. Hence, this template was used by Wellcome Research Laboratories as a "lead" for synthesis of antiparasitic clinical agents, namely, parvaquone and buparvaquone, as veterinary drugs for the treatment of theileriosis in cattle, while atovaquone has been formulated as an antimalarial (Malarone) approved for human use [90].

The antitumor activity of lapachol was documented as early as the 1970s, when it was found to inhibit tumors in rodent models [91]. Later on, studies were carried out to confirm the antimetastasis activity of this compound [92]. Recent experiment on murine Ehrlich carcinoma and human K562 leukemia cells showed the partial DNA- topoisomerase II inhibitory activity of lapachol [93].

34.3.4.1 HPLC Analysis of Lapachol

HPLC analysis of lapachol content in the inner bark of *T. avellanedae* (Bignoniaceae) was reported by Steinert et al. [94], by using a liquid chromatograph (Model 2249 LKB Pharmacia, Bromma, Sweden) fitted with a Rheodyne injection valve connected to a stream of nitrogen. The plant extract was taken up in methanol:tetrahydrofuran (4:1, v/v) and, after membrane filtration, applied on a Spherisorb ODS-2 column (Herrenberg, Germany; 5 μm particle size; 250 × 4 mm i.d.), set up with a UV-VIS detector at 254 nm. The mobile phases consisted of water:methanol:acetonitrile mixtures in various ratios, and the water was always acidified with phosphoric acid. Subsequently, the same group of workers described a RPLC method for analysis of 13 quinonoid constituents, including lapachol, from the heartwood of *T. avellanedae* [95]. Incidentally, lapachol was found to be a minor constituent in the inner bark, while it was more predominant in the heartwood of this plant. HPLC was performed with the same equipment, using gradient elution with mobile phases consisting of water:methanol:methyl-*tert*-butylether (MTBE) mixtures. The separation of most of the quinonoid constituents, including the isomeric α- and β-lapachone, could be achieved in ~20 min. According to the authors, this resolution was possible by adding the ether in the mobile phase: methanol:water:H_3PO_4:MTBE for a gradient elution process. Further, it was important to use an endcapped ODS column in order to prevent the tailing of hydroxyquinones despite the presence of acidified eluents, keeping in view the basic nature of the Spherisorb silica used in the separation column.

Recently, a high-speed countercurrent chromatography (HSCCC) technique has been developed for separation and purification of lapachol and β-lapachone from the reaction mixture during

synthesis of their heterocyclic derivatives [96]. This equipment involving liquid–liquid extraction would help to overcome the degradation of products on the solid phase of silica gel and also save a large amount of time and solvents spent in traditional HPLC columns.

34.3.5 Phylloquinone (Vitamin K_1)

The story of the discovery of vitamin K (Figure 34.1) began as early as 1929, when the Danish scientist Henrik Dam started investigating the role of cholesterol in dietary food. He fed chickens a cholesterol-depleted diet, and they eventually developed hemorrhages after a few weeks and could not be restored even by the addition of purified cholesterol to the meal. Soon after that, an antihemorrhagic quinonoid constituent was identified in food, which was termed the *coagulation vitamin* [97]. The new vitamin received the letter K because the initial discoveries were reported in a German journal, where it was designated as *Koagulationsvitamin*. Edward Adelbert Doisy of Saint Louis University discovered the structure and chemical nature of vitamin K [98] and shared the Nobel Prize for medicine with H. Dam in 1943 for their work on vitamin K.

Vitamin K is a generic name for a group of quinonoid compounds with a methylated naphthoquinone structure, including the naturally occurring forms, (phylloquinone and menaquinones) and a synthetic form (menadione). Dietary vitamin K_1 or phylloquinone is ubiquitously distributed in plants, green leafy vegetables, vegetable oils, and algae and is the main source of vitamin K in the human body [99]. This group of lipophilic vitamins, mostly required for blood coagulation, also plays important roles in bone metabolism, calcification, and vascular health. In epidemiological studies, low dietary intake of vitamin K was associated with an increased incidence of hip fracture, while neonatal and infantile deficiency of this vitamin could cause melena neonatorum and intracranial hemorrhagic diseases [100].

34.3.5.1 HPLC Analysis of Phylloquinone (Vitamin K_1)

HPLC analysis has long been used to study bioavailability of nutrients in animal tissues to determine the health impact of those nutrients/vitamins from foods. Electrochemical or fluorescence detection (after reduction to the hydroquinone form) offers the requisite sensitivity and selectivity for analyzing small amounts of the analyte isolated from the plasma, serum, or tissue of the human or animal subjects after feeding them with the specific food, vegetable, or algae containing vitamin K. To mention a few early reports, Hirauchi et al. [101] measured the K vitamins by HPLC with fluorimetric detection, while electrochemical detection was used for assay of phylloquinone in plasma by McCarthy et al. [102]. A method based on RP-HPLC using postcolumn derivatization and fluorescence detection, combined with a liquid–liquid sample cleanup, was described by Jakob and Elmadfa [103]. A C_{18} column was followed by a short column filled with zinc powder, which was eluted with a nonaqueous solvent system consisting of methanol and dichloromethane. The LOD was found to be 0.04 ng/mL (0.09 nmol/L) for samples of 1 mL plasma. Further, samples covering a wide range of matrices, from those with fairly low (e.g., human plasma and milk) to those with high phylloquinone content (e.g., broccoli), were tested satisfactorily.

Similarly, in another RPLC method, a triacontyl-bonded C_{30} column was used, followed by postcolumn reduction to the fluorescent hydroquinone derivatives [104]. Lipids were removed by lipase digestion, followed by single extraction into hydrocarbon, and the protocol was extended to selected natural and processed foods. This method could successfully measure the amounts of biologically active *trans*- and inactive *cis*- isomers of vitamin K_1, individually, to evaluate the true nutritional status of products, such as milk and infant formula as well as a wider range of foods important in the human diet. The inactive *cis*-vitamin K_1 isomer contributed up to about 15% of total phylloquinone in certain foods. A recent survey was conducted in Japan to estimate the dietary intake of vitamin K by young women. Several food items, such as cereals, vegetable, algae, fish, meat, and so on, consumed in the diet were estimated for vitamin K_1 content by using a platinum reduction column (15×4 mm) placed between the HPLC column(C_{18}; 250×4.6 mm; Shimadzu) and the fluorescence

detector. The mobile phase for estimation of phylloquinone was a mixture of methanol:ethanol (5:5, v/v) [105].

Recently, conditions have been standardized for analyzing the pigment composition in marine algae with a high vitamin K content by using HPLC with a polyether ether ketone (PEEK; 150 × 4.4 mm i.d.) column packed with Pegasil ODS (Senshu Science, Tokyo). Three detectors, specifically two UV-VIS and one PDA detector, were connected in series to monitor the mixture of pigments eluted from the cyanobacterium extract [106]. Another method was developed for the determination of vitamin K homologues, including phylloquinone, in human plasma, using postcolumn peroxyoxalate chemiluminescence (PO-CL) detection following on-line ultraviolet (UV) irradiation (254 nm, 15 W) of vitamin K to produce hydrogen peroxide and a fluorescent product at the same time, which was determined with PO-CL detection. The separation was accomplished isocratically on an HPLC ODS column (Develosil UG-5; 50 × 1.5 mm i.d., Nomura Chemicals, Tokyo). A mixture of imidazole:HNO_3 buffer (600 mM, pH 9.0) and acetonitrile (5:95, v/v) was used as a mobile phase and 0.6 mM bis [2-(3,6,9-trioxadecyloxycarbonyl)-4-nitrophenyl] oxalate (TDPO) in acetonitrile was used as the postcolumn CL reagent [100].

Clinical assessment of phylloquinone status is useful in patients with obstructive liver disease, malabsorption due to celiac disease, or pancreatitis, and its measurement is analytically demanding because this quinone is present in concentrations as low as one nanomole per liter in plasma. Thus, it was extracted from fasting plasma samples by deproteinization and C_{18} solid-phase extraction, separated by reversed-phase HPLC, and detected fluorometrically after postcolumn reduction with a platinum catalyst. The analysis was done on an RPLC column (5 μm Luna C_{18}; 250 × 4.6 mm i.d.), and the isocratic mobile phase was a mixture of methanol:ethanol (80:20, v/v) [99].

34.3.6 Alkannin and Shikonin

The history of alkannin and shikonin (Figure 34.1), the enantiomeric isohexenylnaphthazarins, has its origin in two continents, progressing concurrently up to the 20th century. The *S*-enantiomer (alkannin) is found as a deep red pigment in the roots of *Alkanna tinctoria* Tausch. (Boraginaceae), traditionally used as a textile dye in Europe. Even to this day, alkannin is used as a pigment in food and cosmetics in Europe and North America, while its optical antipode, shikonin (the *R*-enantiomer), is the major constituent of the red pigment extracted from roots of *Lithospermum erythrorhizon* Sieb et Zucc. (Boraginaceae) in China. Documented in the classic compilation of traditional Chinese medicine *Pen T'sao* (1596 A.D.), the use of *L. erythrorhizon* roots for burns, ulcers, sores, wounds, hemorrhoids, and dermatitis is markedly similar to the medicinal properties claimed for *A. tinctoria* in *De Materia Medica* by Dioscorides in Greece (77 A.D.). Currently, roots of *Lithospermum* and *Arnebia* spp. are formulated as traditional drugs popular in Japan (Ko-Shikon; Nan-Shikon), China (Shi-Ka-Ron; Zicao), and India (Ratanjot). However, alkannin and shikonin could also be isolated from the genera *Echium*, *Onosma*, *Anchusa*, and *Cynoglossum* of the same Boraginaceous family. The ratio of the two enantiomers was found to vary with the respective species and could be determined by circular dichroism or chiral stationary-phase HPLC [107].

Biological studies on the chiral pair, alkannin and shikonin, established a wide spectrum of antimicrobial [108], anti-HIV [109], antioxidant [110], and anti-platelet-aggregatory [111] activity. Substantial studies were undertaken on the anti-inflammatory [112] property associated with the inhibition of TNF-alpha promoter [113], and iNOS enzyme [114]. A number of experiments confirmed the potential anticancer [115,116] and anti-angiogenic [117,118] efficacy of shikonin and its analogues. A mechanistic study revealed that the cytotoxic potential of these compounds involved their ability to induce apoptosis by DNA topoisomerase inhibition [51,119], and also through the activation of p53, p27, coordinative modulation of Bcl-2 family, release of cytochrome c, and sequential activation of caspases [120,121], with decreasing phosphorylation of EGFR, ERK1/2, and protein tyrosine kinases and increasing phosphorylated JNK1/2 levels in cancer cell lines [122].

34.3.6.1 HPLC Separation of Alkannin and Shikonin

HPLC separation of alkannin and shikonin from roots of Boraginaceous species was reported in early 1980s by Tsukada, Nickel, Zu, and some others [123]. Reportedly, the first chromatographic enantioseparation was established in 1991, when Ikeda and coworkers used chiral-phase HPLC with a Chiralcel OD column (250 × 4.6 mm) eluted with *n*-hexane:isopropanol (90:10, v/v), and UV-VIS detection at 520 nm [124] to isolate alkannin and shikonin from the root/callus extracts of *Lithospermun eryhtroshizon*, *Arnebia euchroma*, and *Alkanna tinctoria*. Subsequently, Yesilada et al. [125] used the same Chiralcel OD column for quantitive determination of alkannin and shikonin in 18 Boraginaceous root samples growing in Turkey. Kang et al. [126] followed with a similar method applied to Lithospermi radix. Detailed analytical studies were performed by Assimopoulou and Papageorgiou [127], who used a chiral Kromasil column for the first time for enantioseparation of alkannin and shikonin, as well as for detection of their mono- and polymeric derivatives co-occurring in the plant samples. This column was found to be more sensitive for resolution of these compounds compared to the ones used in earlier methods [128]. The same group of authors have developed size-exclusion chromatography to determine the oligomeric degradation products that adversely affect the quality of pharmaceutical preparations containing alkannin and shikonin.

In 2006, Hu et al. [129] used diode array detection (Agilent/HP 1100 series; C_8 column; 250 × 4.6 mm i.d., 5 μm) for the simultaneous quantification of eight naphthoquinone derivatives, viz. shikonin, acetylshikonin, deoxyshikonin, β-acetoxyisovalerylshikonin, isobutylshikonin, β,β-dimethylacrylshikonin, 2-methyl-*n*-butyrylshikonin, and isovalerylshikonin, found in nine species of the Boraginaceae family. These species, coming from different areas of China, have been used as interchangeable sourcing plants for the Chinese "Zicao"; they are *Arnebia euchroma* (Royle) Johnston., *A. guttata* Bunge, *Lithospermum erythrorhizon* Sieb. et Zucc., *Onosma paniculatum* Bur. et Franch., *O. exsertum* Hemsl., *O. confertum* W.W. Smith, *O. hookerii* Clarke var. *longiflorum* Duthie, *O. hookerii* Clarke, and *O. waltonii* Duthic. The mobile phase consisted of (A) water:formic acid (100:0.2, v/v) and (B) methanol:tetrahydrofuran (100:5, v/v) with a gradient program.

34.3.7 Diospyrin

Diospyrin (Figure 34.1) is the most abundant naphthoquinonoid found in *Diospyros montana* Roxb.; it is also present in several other *Diospyros* and *Euclea* spp. The stem bark, root, leaf, fruits, and seeds of *Diospyros* spp. have been utilized in ethnomedical formulations in Asia and Africa, mostly as an astringent chewing stick, in the treatment of urinary, skin, and abdominal diseases, gynecological problems, schistosomiasis, spleen inflammation, snake bites, and so on. In India, *D. melanoxylon* leaves are commercially valuable for wrapping country cigarettes ("bidi") [2].

The tumor-inhibitory activity of *Diospyros montana* stem bark was observed in early 1980s, followed by identification of diospyrin as the bioactive principle [130]. Thereafter, this naphthoquinonoid was found to inhibit the growth of the *Leishmania donovani* parasite [131], and the therapeutic prospects of this natural product were improved through synthesis of suitable derivatives [132–134]. Studies were undertaken in order to elucidate the antibacterial [32], antitubercular [135], antimalarial [132], and anticancer [136–139] properties of the novel group of quiononoids derived from diospyrin.

34.3.7.1 HPLC of Diospyrin

The quantification of diospyrin in the chloroform extract of the stem bark of *Diospyros montana* by the LC-UV method was first reported from our laboratory [62]. The stem bark samples of *D. montana* were collected from four different climatic regions of India. LC analysis was performed on a μ-Bondapak C_{18} steel column (300 × 3.9 mm i.d.; particle size 10 μm) under isocratic conditions

(acetonitrile:water, 50:50, v/v), at a flow rate of 1.0 mL/min at ambient temperature, followed by UV detection at 255 nm. The amount of diospyrin was quantitatively estimated within 15 min under these conditions with a LOD of 8 ng and a LOQ of 20 ng.

Joubert et al. [140] reported a comparative HPLC study of the root extracts of eight different *Euclea* species. Five of those in South Africa (*E. crispa*, *E. divinorum*, *E. natalensis*, *E. pseudebenus*, and *E. undulata*), showed the presence of diospyrin.

34.4 ANTHRAQUINONOID PHYTOCHEMICALS

Anthraquinonoid phytochemicals comprising emodin and its analogues, namely, aloe-emodin, rhein, chrysophanol, and physcion (Figure 34.1), are major constituents of many plants traditionally used in TCM and other Oriental medicinal formulations. Again, alizarin and its analogues, such as purpurin, lucidin, quinizarin, and munjistin (Figure 34.1), mostly occur in Rubiaceous plants and are useful as coloring agents.

34.4.1 Emodin and Analogs

Five hydroxyanthraquinonoid analogues (aloe-emodin, rhein, emodin, chrysophanol, and physcion; Figure 34.1) are major bioactive constituents of the genera *Aloe*, *Rheum*, *Polygonum*, *Cassia*, and so on. Many traditional Oriental herbal formulations of the roots, rhizomes, stems, and/or leaves of these species continue to be popular even to this day, with a lot of commercial prospect. Hence, the analysis and standardization of these five major analogues have drawn the attention of many workers in this field, and the early reports on HPLC separation are compiled in Table 34.1.

Most of these herbal preparations have been used all over the world for their laxative and purgative property. In Southeast Asian countries and India, *Aloe vera* was popular for its abortifacient, uterine stimulant, cathartic, emmenagogue, cholerectic, and anthelmintic properties. The gel of this plant was applied for healing open wounds and sores. The rhizome of common rhubarb (*Rheum officinale* and *R. palmatum*) is one of the chief constituents in the ancient Japanese kampo formulation for its anti-inflammatory cathartic, and antipsychotic properties. Similarly, *Polygonum cuspidatum* has also been traditionally utilized in China for hepatitis, skin burns, inflammation, and osteomyelitis [27].

Emodin and its analogues were found to possess antibacterial [141], antifungal [142], anti-inflammatory [143], immunosuppressive, and estrogenic [144,145] properties. The tumor-growth-inhibitory activity of emodin analogues and related mechanisms, chiefly associated with the induction of programmed cell death, are also currently under rigorous investigation [146,147]. Presumably, the worldwide ethnomedical application of the previously mentioned herbal preparations is associated with the ubiquitous presence of emodin and its analogues in the constituent plants.

34.4.1.1 HPLC Analysis of Hydroxyanthraquinone Derivatives

HPLC analysis of the five hydroxyanthraquinone derivatives in *Rheum emodi*, *Rheum officinale*, *Polygonum cuspidatum*, and *Cassia alata* collected from different places indicated locational variations in the amounts of the quinonoids in the plant samples [2] (Table 34.1). For example, methanolic extracts of rhizomes of *Rheum emodi* collected from three locations of in the western Himalayas in Himachal Pradesh, India, were subjected to LC-PDA analysis by Verma et al. [148] using a Purospher-Star RP-18e column (250 × 4.6 mm i.d., 5 μm) under the following conditions: solvent A, acetonitrile:methanol (95:5, v/v), and solvent B, water:acetic acid (99.9:0.1, v/v), as the mobile phase with linear gradient elution at a flow rate of 0.8 mL/min. The detection wavelength was set at 290 nm. Thus, separation of emodin, chrysophanol, and physcion, along with glycosides of emodin and chrysophanol, was achieved with detection limits ranging from 0.56 to 3.50 ng/mL. Similarly, Komatsu et al. [149] analysed 24 rhubarb samples, including *Rheum tanguticum*, *R. palmatum*, and *R. officinale*, using RPLC with a PDA detector. An Inertsil ODS column (250 × 4.6 mm i.d.;

5 μm; GL Science Inc.) was used with gradient elution, either with a mixture of 0.05 M H_3PO_4 and acetonitrile or a mixture of 0.05 M H_3PO_4, acetonitrile, and methanol.

Recently, the simultaneous separation of 11 anthraquinones from rhubarb, including emodin, chrysophanol, rhein and its glucosides, aloe-emodin, sennoside A, and sennoside B has been achieved by application of both HPLC and capillary electrophoresis (CE), thereby presenting a comparative study of these two separation techniques [150]. The results showed that the detection limits of anthraquinones from rhubarb through HPLC were lower than those for CE (HPLC: 0.02–0.2 μg/mL; CE: 0.1–0.8 μg/mL), though both the methods provided acceptable analysis times (except for rhein in CE, which was > 28 min). Therefore, it was difficult to consider one of these methods more efficacious than the other. HPLC was conducted by varying the column length (Cosmosil 5C18ARII; Nacalai Tesque, Kyoto, Japan) and operating temperature (in the range of 28–60°C) and using a UV-PDA detector at 254 nm with a gradient elution with acetonitrile and water.

Very recently, a reversed-phase ultra performance liquid chromatography (UPLC) method was developed for the rapid quantification of five anthraquinone derivatives (aloe-emodin, rhein, emodin, chrysophanol, and physcion) in rhubarb using a Waters Acquity BEH C_{18} column (50 × 2.1 mm, 1.7 μm; Figures 34.2 and 34.3). In fact, using sub-2 μm particles for the stationary phase and a shorter column length, UPLC provided equivalent resolution and faster chromatographic separations. Because of its speed and sensitivity, this technique would be useful for biomedical analysis, and also for routine standardiation of botanical drugs consisting of multiple components. This method was found to be reproducible and conveniently applicable to three species of rhubarb samples, namely, *Rheum palmatum*, *R. tanguticumi*, and *R. officinale* (Figure 34.2), and to a couple of Chinese medicinal preparations containing these herbs, namely, Sanhuang tablet and Paidu Yangyan capsule. By applying UPLC, the elution time was found to be reduced by almost 8-fold (~3 min) in comparison to conventional HPLC (~24 min; Figure 34.3).

The first investigation on the simultaneous analysis of these five anthraquinonoids in *Cassia alata* root was reported by Fernand et al. [151]. The root extracts were purified by solid-phase extraction on C_{18} cartridges, followed by LC–UV–atmospheric pressure chemical ionization (APCI)–MS on a C_{18} RP column, using an isocratic mobile phase of acetonitrile, methanol, and 10mM aqueous ammonium acetate buffer at pH 6.8 (25:55:20, v/v). LOD values for these quinonoids were observed

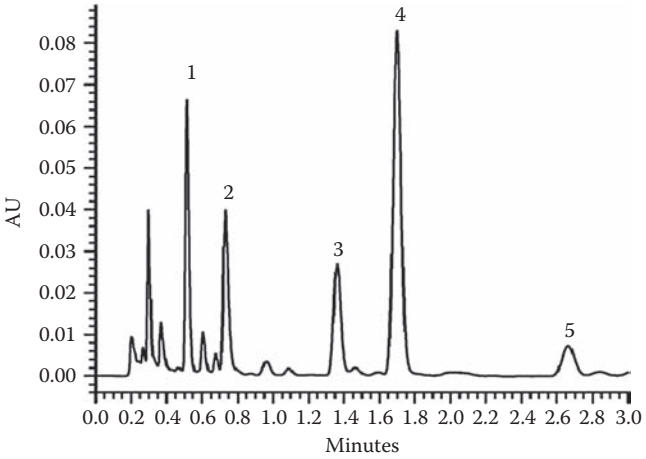

FIGURE 34.2 Ultra performance liquid chromatography–ultraviolet detection (UPLC-UV) chromatogram of crude sample of *Rheum palmatum*. Mobile phase: 0.1% aqueous phosphoric acid:methanol (31:69, v/v) in isocratic mode; flow rate: 750 μL/min at 35°C; UV detection at 254 nm. Peaks: 1 = Aloe-emodin (0.546 min); 2 = Rhein (0.788 min); 3 = Emodin (1.487 min); 4 = Chrysophanol (1.849 min); 5 = Physcion (2.751 min). (Reproduced from Wang, J., Li, H., Jin, C., Qu, Y., and Xiao, X., *J. Pharm. Biomed. Anal.*, 47, 765–770, 2008. With permission from Elsevier Science.)

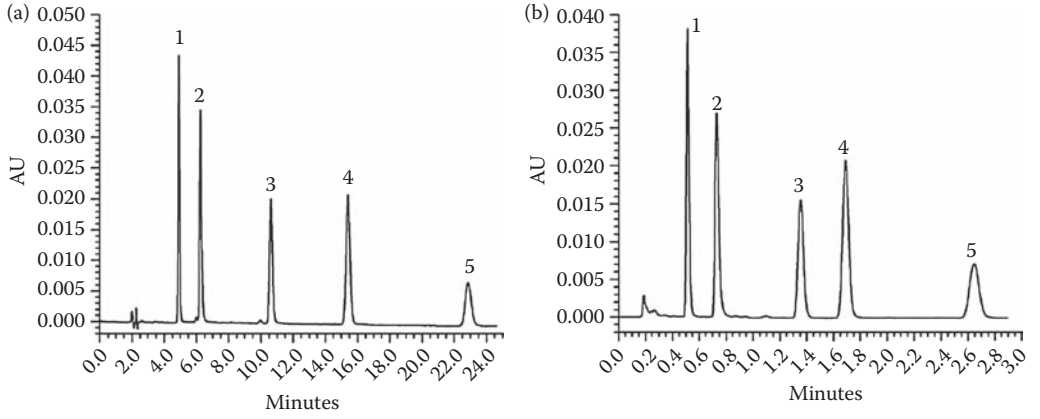

FIGURE 34.3 Comparison of chromatograms of mixed standard obtained by (A) high performance liquid chromatography (HPLC) and (B) ultra performance liquid chromatography (UPLC). For HPLC: Mobile phase: 0.1% aqueous phosphoric acid:methanol (15:85, v/v) in isocratic mode; flow rate: 1 mL/min at 25°C; PDA detection at 254 nm. Peaks: 1 = Aloe-emodin (4.907 min); 2 = Rhein (6.252 min); 3 = Emodin (10.622 min); 4 = Chrysophanol (15.402 min); 5 = Physcion (22.861 min). For UPLC: Mobile phase: 0.1% aqueous phosphoric acid:methanol (31:69, v/v) in isocratic mode; flow rate: 750 µL/min at 35°C; UV detection at 254 nm. Peaks: 1 = Aloe-emodin (0.546 min); 2 = Rhein (0.788 min); 3 = Emodin (1.487 min); 4 = Chrysophanol (1.849 min); 5 = Physcion (2.751 min). (Reproduced from Wang, J., Li, H., Jin, C., Qu, Y., and Xiao, X., *J. Pharm. Biomed. Anal.*, 47, 765–770, 2008. With permission from Elsevier Science.)

in the range of 0.23 ppm (for emodin) to 4.61 ppm (for rhein). Another study involving LC–PDA–electrospray ionization (ESI)–MS for analysis of the same group of quinonoids in *Polygonum cuspidatum* rhizomes reported high sensitivity, with LODs in the range of 0.51 ng (for aloe-emodin) to 0.82 ng (for physcion) [152].

Rapid simultaneous quantification of five active constituents (including rhein, emodin, and aloe-emodin) from *Da-Cheng-Qi* decoction (DCQD), a famous purgative from the Chinese medicinal formula, in rat plasma has been validated by an LC-ESI-MS/MS assay [153]. A single dose of DCQD, containing the root and bark of *Rheum palmatum*, was administered orally to Sprague–Dawley rats, and blood was collected over a period of 48 h. Each plasma sample was eluted on a RPLC C_{18} column packed with smaller particles (3.5 µm; 100 × 3.0 mm) using a mobile phase of methanol:0.1% formic acid aqueous solution (70:30, v/v). The analytes of aloe-emodin, rhein, and emodin were eluted rapidly in less than 8 min (Figure 34.4).

34.4.2 Alizarin and Analogs

Numerous hydroxyanthraquinones, consisting mainly of alizarin, lucidin, purpurin, quinizarin (Figure 34.1), and their analogs—free or in the form of glycosides—are present in the roots of other rubiaceous plant sources: *Galium* spp., *Oldenlandia umbellata*, *Asperula tinctoria*, *Asperula ciliata*, *Crucianella maritima*, *Hedyotis auricularia*, *Morinda umbellata*, *Morinda citrifolia*, and so on [154].

Historical reference to the coloring agents from the common madder plant (*Rubia tinctorum* and other *Rubia* species) goes back to the findings of stained cloth in the tomb of the Pharaoh Tutankhamen, and also in the ruins of Pompeii and ancient Corinth. The roots and rhizomes of the Rubiaceae family, such as *Rubia tinctorum* (common madder plant), were known in the past as rich sources of coloring materials, especially for red dyes [155]. Alizarin (derived from the Arabic word *al-usara*, "juice"), the main coloring component identified in madder root, was the first natural pigment synthesized chemically in the early 19th century. In 1826, the French chemist Pierre-Jean

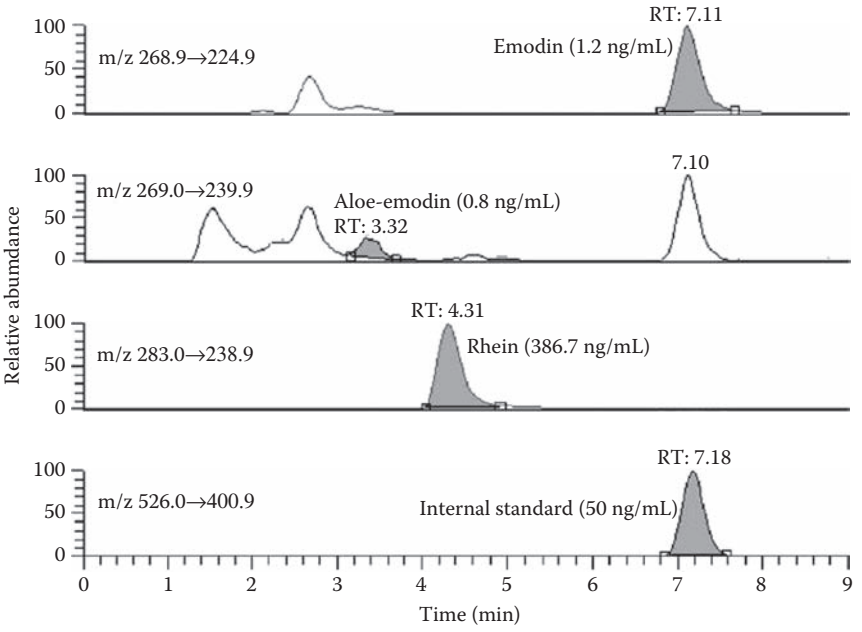

FIGURE 34.4 The selected reaction monitoring liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) chromatograms of plasma samples of Sprague–Dawley rats obtained at 0.5 h after oral administration of *Da-Cheng-Qi* decoction. Mobile phase: methanol:0.1% aqueous formic acid solution (70:30, v/v) in isocratic mode; flow rate: 0.4 mL/min. (Reproduced from Xu, F., Liu, Y., Zhang, Z., Song, R., Dong, H., and Tian, Y., *J. Pharm. Biomed. Anal.*, 47, 586–595, 2008. With permission from Elsevier Science.)

Robiquet found that madder root contained at least one more anthraquinone colorant, purpurin, which faded out more rapidly than alizarin. Even today, the pigments obtained from various *Rubia* species are used for dyeing fabrics, and also to impart color to foods (candies, chewing gums, noodles, jams) and cosmetics (hair dyes, shampoos) [156].

Besides its use as a dyeing component, *Rubia* was utilized as one of the major constituents of traditional medicinal formulations in India and China for treating ailments, like arthritis, sores and wounds, dysmenorrhea, ulcers, leprosy, hepatic obstruction, indigestion, jaundice, paralysis, snake poison, and scorpion bites [27]. To rationalize such ethnomedical claims, evaluation of the medicinal properties of alizarin, purpurin, and their analogs has been started rather recently. The anticancer activity of purpurin used in combination with light, inducing apoptotic cell death in the human leukemia cell line (HL60), was reported [157]. Alizarin is used as a staining agent of bones for scrutinizing the calcification and skeletal development [158–160] and as an efficient colorant for detection of protein [161].

34.4.2.1 HPLC Analysis of Alizarin and Analogs

Several methods for HPLC analysis of *Rubia tinctorum* roots have been reported [162–164], although total baseline separation of glycosylated anthraquinones could not be achieved during simultaneous detection of the anthraquinones and their glycosides [164]. However, Derksen et al. [165] succeeded in quantitative detection of two glycosylated anthraquinones (lucidin primeveroside and ruberythric acid), along with five aglycones, in an extract of *R. tinctorum* in a single run, using an endcapped C_{18} RP column for linear gradient elution with a water:acetonitrile mixture and PDA detection at 250 nm (Figure 34.5). The same group of workers subsequently developed an improved LC-PDA-MS method with on-line mass detection, using APCI as well as ESI, for the rapid characterization of 16 such anthraquinones (Figure 34.6) [166]. In this method, gradient

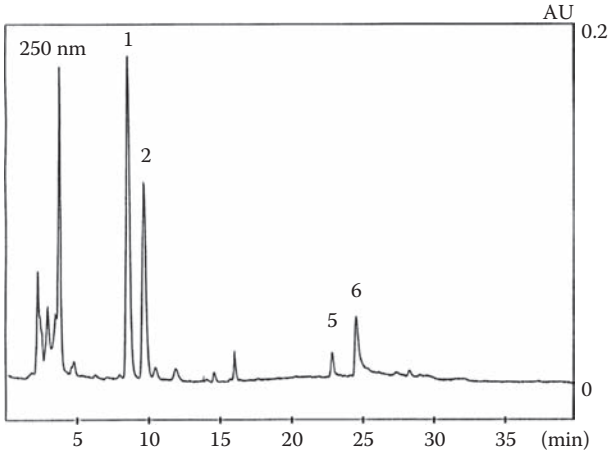

FIGURE 34.5 Liquid chromatographic–photodiode array (LC-PDA) chromatogram of a crude extract of *Rubia tinctorum* roots. Mobile phase: solvent A (water) and solvent B (acetonitrile) in gradient mode; flow rate: 1.0 mL/min; PDA detection at 250 nm. Peaks: 1 = Lucidin primeveroside (8.1 min); 2 = Ruberythric acid (9.1 min); 5 = Alizarin (23.8 min); 6 = Purpurin (25.8 min). (Reproduced from Derksen, G.C.H., van Beek, T.A., de Groot, Æ., and Capelle, A., *J. Chromatogr. A*, 816, 277–281, 1998. With permission from Elsevier Science.)

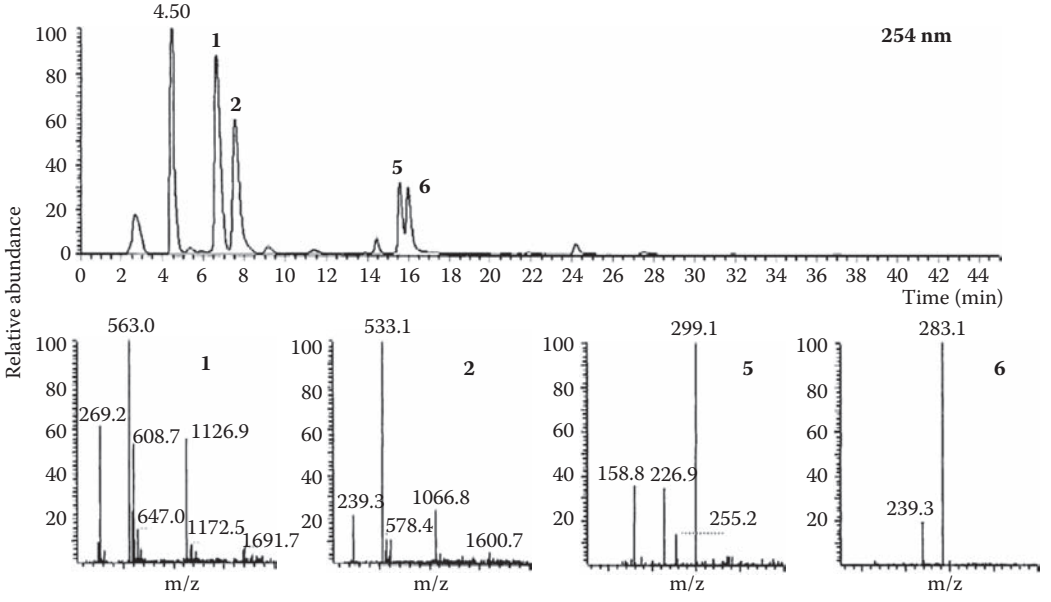

FIGURE 34.6 Liquid chromatographic–photodiode array–mass spectrometry (LC-PDA-MS) analysis of a crude extract of *Rubia tinctorum* roots. Mobile phase: solvent A (ammonium formate-formic acid buffer, 0.2 M, pH 3, and EDTA, 30 mg/L) and solvent B (acetonitrile) in gradient mode; flow rate: 1.0 mL/min; UV detection at 254 nm. MS peaks (negative ion-electrospray ionization (NI-ESI) with post-column addition of ammonia) for individual anthraquinones: 1 = Lucidin primeveroside; 2 = Ruberythric acid; 5 = Pseudopurpurin; 6 = Munjistin. (Reproduced from Niederlander, H.A.G., and van Beek, T.A., *J. Chromatogr. A*, 816, 277–281, 1998. With permission. *J. Chromatogr. A*, 978, 119–127, 2002. With permission from Elsevier Science.)

elution with a mobile phase composed of (A) ammonium formate-formic acid buffer (0.2 M; pH 3) with EDTA (30 mg/L) and (B) acetonitrile was used. In a continuation of their effort, Derksen et al. developed two new methods to quantify anthraquinones in madder roots by screening of cultivars of this species. In the direct method, madder root was extracted twice with refluxing ethanol:water for 2 h, leading to the determination of the two major native anthraquinone glycosides, namely, lucidin primeveroside and ruberythric acid. In the indirect extraction method, the anthraquinone glycosides were first hydrolyzed by endogenous enzymes, and the aglycones were subsequently extracted with tetrahydrofuran:water:formic acid for analysis by HPLC-UV methods [167].

Samples of dyed fibers from ancient objects were subjected to analytical protocols to identify the origin of the natural dye used. Thus, objects originating from fourth- to twelfth-century Egypt and belonging to the collection of early Christian art at the National Museum in Warsaw were examined by LC-PDA [168,169]. Extraction of the natural dyes from fibers was carried out with HCl solution containing ethanol or with warm pyridine, when mainly anthraquinones, including alizarin and purpurin, were found to be the chemical components. Similar studies were carried out by the LC-UV-MS method [169]. Here, the detection threshold is a problem, as samples originating from archaeological and historical objects are often tiny, and the dyes in artistic paintings are very often deteriorated. Usually, identification based on PDA would be limited to commercially available compounds as standards. Therefore, detection by fluorescence spectroscopy and MS has been developed over the last few years, and necessary optimization was carried out using source parameter step-adjustment, mobile-phase composition, and postcolumn additive testing. In fact, LC-MS could be finely tuned to enable both detection and characterization, showing advantages in comparison to PDA, with improved molecular identification and better selectivity [154]. Again, as several compounds had the same molecular mass and MS^3 fragmentation, their retention parameters were useful for adequate structure recognition. The optimized conditions were successfully applied to the identification of components from a small sample of wool thread dyed with madder (*Rubia tinctorum*). Thus, the identification and quantification of components of natural products used in art helped in restoration and conservation procedures.

34.5 CONCLUSION

In conclusion, it suffices to say that quinonoid phytochemicals have generated a spurt of interest, particularly in view of the new revelations about their potent biological activities and relevant mechanisms of action. This is apparent from the overwhelming number of reports currently appearing in the literature on adaptation of existing HPLC methods to specifically detect or estimate this type of compounds in herbal medicinal formulations and biological samples. In fact, some of the quinonoids, like shikonin and emodin analogs, deserve more extensive reviews to cover the ongoing progress in this field.

ACKNOWLEDGMENTS

The authors are grateful for research grants received from the University Grants Commission, New Delhi, Department of Atomic Energy, BRNS, Bhabha Atomic Research Centre, Mumbai, and Council of Scientific and Industrial Research, New Delhi.

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Index

A

B

C

D

G

H

K

L

M

O

Q

R

U

High Performance Liquid Chromatography in Phytochemical Analysis

High Performance Liquid Chromatography in Phytochemical Analysis is the first book to give a complete description of the techniques, materials, and instrumentation of column high performance liquid chromatography (HPLC) and its application to essentially all primary and secondary plant metabolites. Hailing from around the world and with vast expertise in HPLC phytochemical analysis, the contributors present a global, authoritative view of the field.

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ISBN: 978-1-4200-9260-8
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