

Methods of Analysis for Functional Foods and Nutraceuticals

EDITED BY

W. Jeffrey Hurst



FUNCTIONAL FOODS AND NUTRACEUTICALS SERIES

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Methods of Analysis for Functional Foods and Nutraceuticals

FUNCTIONAL FOODS AND NUTRACEUTICALS SERIES

Series Editor

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Functional Foods: Biochemical and Processing Aspects

Volume 1

Edited by G. Mazza, Ph.D.

Herbs, Botanicals and Teas

Edited by G. Mazza, Ph.D., and B.D. Oomah, Ph.D.

Functional Foods: Biochemical and Processing Aspects

Volume 2

Edited by John Shi, Ph.D., G. Mazza, Ph.D., and Marc Le Maguer, Ph.D.

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Series Preface

The Functional Foods and Nutraceuticals Series was developed to offer food, nutrition, and health practitioners a comprehensive treatment of the emerging science and technology of functional foods and nutraceuticals shown to play a role in preventing or delaying the onset of diseases, especially chronic diseases. Books in the series cover a wide range of developments in chemistry, biochemistry, pharmacology, epidemiology, and engineering of products from plants and animal sources; results of animal and clinical trials; and regulatory, standardization, and quality control issues.

This volume, *Methods of Analysis for Functional Foods and Nutraceuticals*, edited by W. Jeffrey Hurst, presents advanced methods of analysis for carotenoids, phytoestrogens, chlorophylls, anthocyanins, amino acids, fatty acids, flavonoids, water-soluble vitamins, and carbohydrates. Dr. Hurst has assembled a stellar list of international contributors in the forefront of analysis of phytochemicals.

It is hoped that the effort will be useful to food, nutrition, and health practitioners and to students and researchers in industry, government, and university laboratories.

G. Mazza
Series Editor

Preface

A book on the analysis of functional foods should first define “functional foods.” Unfortunately, there are numerous definitions currently being used, which can result in substantial confusion. For the purposes of this book, functional foods are defined as foods that are similar in appearance to or may be conventional foods that are consumed as part of a usual diet and are demonstrated to have some physiological benefits and/or reduce the risk of diseases beyond basic nutritional functions. In some of the chapters in this book, you will notice that the author has chosen to provide his or her own perspective on this terminology, reflecting the diversity seen by authors from around the globe.

This volume is the fourth in the Functional Foods and Nutraceuticals Series. It is not a general food analysis book, since there are presently a sufficient number of those in print. It was developed to concentrate on the analytical aspects of functional foods, with a focus on a number of compound classes. You will notice that there are subjects that have not been included, for a variety of reasons. For example, we have made a conscious decision not to include a chapter on added botanicals, since that topic has been included in the second book in this series, *Herbs, Botanicals and Teas*, edited by G. Mazza and D. Oomah and would have been superfluous to this offering. Each chapter in this book focuses on a particular compound class rather than a food type, since this will allow an analyst the opportunity to see the determination of a particular compound in a variety of food matrixes rather than be limited to a single matrix. The author of each chapter has provided an overview of the particular topic with information on a variety of methods to allow for the determination of members of that compound class in food matrixes. Additionally, each chapter, while important to the overall theme of the book, was written to be able to stand independently from the others.

The nine chapters were carefully chosen so that each reflects an important area of interest and research in functional foods. The book includes a chapter on phytoestrogen analysis, which is of increasing interest to the scientific and medical community, especially with respect to the soy isoflavones and their impact on women’s health. There are few resources on the analysis of these compounds. Two chapters discuss the analysis of vitamins, with one focusing on water-soluble vitamins and the other focusing on carotenoids and provitamin A. The determination of vitamins in food has been of interest for decades, and these chapters provide an update on methods along with an emphasis on functional foods. Fatty acid analysis is always a timely topic in any food analysis book, and in our case we have chosen to concentrate on ω 3 fatty acids and conjugated linoleic acid (CLA), since both of these topics are gaining in importance with respect to positive health effects from their consumption. Amino acid analysis was selected for inclusion because these compounds are critical to the makeup of many functional foods. There are chapters

on flavonoids and anthocyanins, both of which are the subject of intense interest and increasing research; on an almost daily basis one can read the scientific and popular press and find an article about some member of these classes, ranging from blueberries to tea, coffee, and cocoa. Moreover, analytical information on these compounds tends to be published not only in mainstream journals, but also in more niche journals not available to all researchers. A chapter on chlorophyll analysis might initially seem out of place here, but in the literature we see an expanding number of citations and, as the authors of the chapter indicate, a growing interest in health effects. The final chapter concentrates on carbohydrates and other electrochemically active components. While there is continued interest in the determination of simple carbohydrates, there is scant information on some of the complex carbohydrates. Additionally, this chapter discusses the application of this technology for the determination of other classes of compounds, such as sulfur compounds, that are of interest in functional foods.

The information included in this volume is timely and appropriate for a large audience, which can include practicing analysts and researchers, those in management interested in this topic, and food science faculty and students. In addition to providing the information noted above, each chapter includes references for further reading.

I thank all who have been involved in this project for their continued interest and support, with the biggest thanks to my wife, Deborah, for her support when I agreed to work on "another book."

W. Jeffrey Hurst

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1 Phytoestrogens

B. Dave Oomah

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1.1 INTRODUCTION

Phytoestrogens are diphenolic compounds that structurally resemble the human sex hormone, estrogen. This shining similarity of these compounds at the molecular level provides phytoestrogens the ability to mildly mimic and in some cases act as an antagonist to estrogen. Phytoestrogens can be grouped into three main classes: coumestans, isoflavones, and lignans. Coumestans are found in red clover and alfalfa, with smaller amounts in split peas and bean sprouts. Isoflavones are found primarily in soybeans and soy products and are probably the best known and intensely investigated for their substantial health benefits among the three classes. Lignans are found in many cereals and grains, with the highest amounts occurring in flaxseed. Despite their more widespread occurrence in foods and their greater consumption in Western populations, the lignans have received comparatively little attention.

Evidence is beginning to accrue that phytoestrogens may begin to offer protection against a wide range of human conditions, including breast, bowel, prostate, and other cancers; cardiovascular disease; brain function; alcohol abuse; osteoporosis; and menopausal symptoms (Bingham et al., 1998). The reported health benefits of the naturally occurring isoflavones, genistein and daidzein, include relief of menopausal symptoms (Nestel et al., 1999; Murkies, Wilcox, and Davis, 1998), reduction of osteoporosis (Gennari et al., 1998), improvement of blood cholesterol levels (Sharma, 1979), and lowering the risk of certain hormone-related cancers (Peterson and Barnes, 1991) and coronary heart disease (*Federal Register*, 1999). The basis for these effects has not been established, but the weak estrogenic activity of isoflavones, sometimes referred to as phytoestrogens, may be a factor in conferring these properties. As a result, many food manufacturers are striving to provide products containing soy and/or isoflavones to consumers.

The interest in phytoestrogens plays an important role in prevention of menopause symptoms, osteoporosis, cancer, and heart disease (Brandi, 1999). Phytoestrogen supplementation with flaxseed or soy flour has been reported to increase vaginal cell maturation, an indication of estrogen activity in postmenopausal women (Wilcox et al., 1990), and to significantly reduce menopause symptom scores, particularly hot flash and vaginal dryness (Brzezinski and Debi, 1999). Dietary studies indicate substantial reduction in breast cancer risk among women with high urinary excretion of phytoestrogens, particularly the isoflavone equol and the lignan enterolactone (Ingram et al., 1997). The lower incidence of prostate cancer in Asian men compared to men from North America and Europe has also been speculated to be due to the higher dietary intake of isoflavones and lignans (Adlercreutz, 1990; Morton et al., 1997).

Phytoestrogens may alter sex hormones and modulate their metabolism at the cellular level by biochemical mechanisms. They influence intracellular enzymes, protein synthesis, growth factor action, malignant cell proliferation and differentiation,

TABLE 1.1
Selected Animal Health-Promoting Effects of Isoflavonoid Compounds

Compound	Effect	Species
Genistein	Prevention of mammary cancer	Rat
Genistein	Inhibition of prostate tumor growth	Human (cell lines)
Genistein, biochanin A	Inhibition of stomach tumor growth	Human (cell lines)
Genistein, daidzein	Activation of natural killer cells	Human
Genistein	Reduction of serum triglycerides and cholesterol	Rat
Genistein, daidzein	Protection against low-density lipoprotein oxidation	Human
Genistein	Prevention of bone loss	Mouse
Genistein	Antiangiogenesis	Human (cell lines)
Daidzin	Suppression of alcohol consumption	Hamster

Source: From Dixon, R. A. and Steele, C. L., *Trends Plant Sci.*, 4, 394–400, 1999. With permission.

angiogenesis, calcium transport, Na^+/K^+ ATPase, vascular smooth muscle cells, and lipid oxidation (Adlercreutz, 1995; Adlercreutz and Mazur, 1997).

Isoflavonoid compounds exhibit a range of mammalian health-promoting activities that are currently the focus of intense study (Table 1.1). Dietary genistein reduces susceptibility to mammary cancer in rats (Fritz et al., 1998) and helps to prevent bone loss caused by estrogen deficiency in female mice (Ishimi et al., 1999). These effects are mirrored by epidemiological evidence that implicates dietary isoflavones as major factors associated with reduced cancer risk in populations consuming a high soy diet (Setchell and Cassidy, 1999). Potential harmful effects have also been shown for isoflavones from forage crops in causing specific infertility problems in sheep feeding on clover (Shutt and Cox, 1972). Their negative role cannot be excluded in the early stage of carcinogenesis (Messina et al., 1994).

These and other studies have provided the impetus for the manufacture of functional foods and nutraceutical products to meet the phytoestrogenic needs of women seeking alternatives to hormone replacement medications. For example, manufacturers in Australia promote the use of commercially produced bread, Burgen® Soy-Lin™ (Chatswood, NSW), that combines soy and flaxseed (Payne, 2000) and claims to contain 220 mg of isoflavones and lignans per four slices (Jorgensen et al., 1998). An example of a nutraceutical in this regard is an isoflavone extract of soybean, 17 β -estradiol (Estrace®, Roberts Pharmaceuticals Canada, Inc., Mississauga, ON), that is being marketed as a prescriptive oral estrogen for effective management of menopause (Oomah and Mazza, 2000). A number of isoflavone extracts are available in pill form as extracts of soy (i.e., Estroven, Healthy Woman, and others) or red clover (Promensil) in doses ranging from 40 to 55 mg/day (Carusi, 2000). The rapid growth of functional foods and interest in the health benefits of plant foods has created the need for standardized methodology for the analysis of phytochemicals, phytoestrogens in particular, in raw and processed products as well as their metabolites in mammalian tissues and fluids. However, investigation of the possible benefits of phytoestrogens is hampered by the lack of analytical standards

and, hence, inadequate methods for the measurement of low levels in most foods. This problem may prove to be a major dilemma for regulatory authorities, clinicians, and others wishing to advise the general public on whether these compounds really do have the health benefits attributed to them. The complexity of phytoestrogens, with at least 15 different chemical forms of the isoflavones alone occurring in foods, is a major limitation in the study of metabolism of these substances in human subjects (Bingham et al., 1998).

1.2 GENERAL ASPECTS

1.2.1 SOURCES

More than 300 plants are known to possess estrogenic activity (Farnsworth et al., 1975), with isoflavones and coumestans (occurring) as the most common estrogenic compounds. The dietary sources of phytoestrogens are only partially known. Oil-seeds, such as flaxseed, have the highest detected contents of lignans. Seaweeds, legumes, cereal brans, whole cereals, vegetables, and fruits contain lesser amounts (Thompson et al., 1991). Rye has a high content of plant lignans that have been hypothesized as one of the factors in rye bran responsible for inhibiting prostate cancer growth (Bylund et al., 2000). Mammalian isoflavonoids are derived mostly from soybeans and fermented soy products. Some alcoholic beverages, such as beer and bourbon, and several varieties of legumes, such as peas and beans, also contain isoflavonoid precursors, but at lower levels (Gavaler et al., 1995; Franke et al., 1994; Lapcik et al., 1998). It has been suggested that resveratrol, a weak phytoestrogen found in grapes and wine, may contribute to the beneficial effects of wine consumption (the "French Paradox") (Kopp, 1998). Resveratrol also exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells (Lu and Serrero, 1999). Similarly, the identification of the potent phytoestrogen 8-prenylnaringenin in hops (*Humulus lupulus*) and beer has been suggested to be a contributing factor in the beneficial health effects of moderate beer consumption (Milligan et al., 1999). Tea contains high levels of lignans, but only low levels of isoflavones (Mazur et al., 1998). Unfortunately, the precise phytoestrogen content of many individual foods is not known and may differ according to variety, location, and season, as has been shown for the isoflavonoid content of soybeans (Eldridge and Kwolek, 1983).

The isoflavonoids, genistein (4',5,7-trihydroxyisoflavone), biochanin A (5,7-dihydroxy-4'-methoxyisoflavone), daidzein (4',7-dihydroxyisoflavone), and formononetin (7-hydroxy-4'-methoxyisoflavone), are supposed to be health-promoting dietary factors of plant origin. They are particularly abundant in seeds and other parts of many plant species belonging to Leguminosae. In the human diet, they are represented by the genera *Glycine*, *Phaseolus*, *Cicer*, *Lens*, and *Pueraria*. In legumes the isoflavonoids are present mainly as 6"-O-malonylglycosides and β -glycosides, and only minimal amounts are present in the aglycone form (Lapcik et al., 1998). The genus *Trifolium* is an important source of phytoestrogens for domestic animals. It has been previously reported that phytoestrogens such as daidzein and genistein are present in crude drugs, including the root of *Puerarie lobata* or the root of *Glycyrrhizae glabra* (Shiizaki et al., 1999). In fact, deoxymiroestrol from *Pueraria*

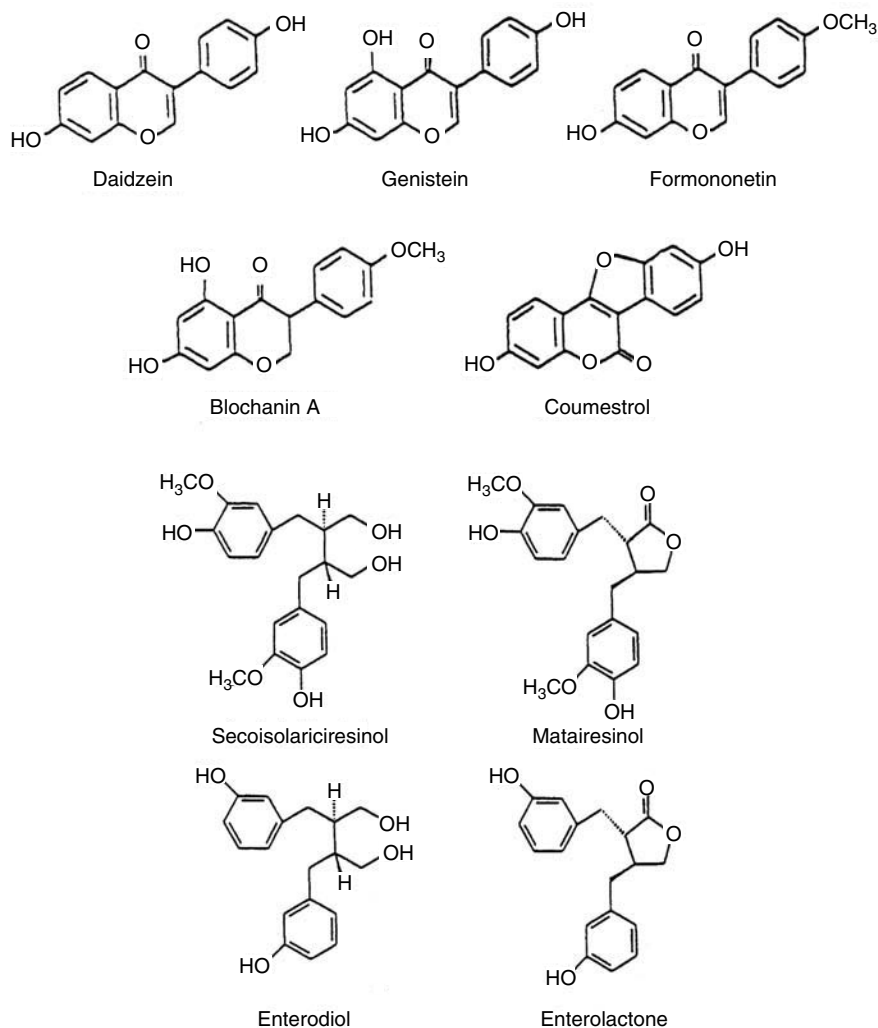


FIGURE 1.1 Structure of common phytoestrogens.

mirifica, a rejuvenating folk medicine from Thailand, is considered to be the compound with the highest estrogenic potency among the known phytoestrogens (Chansakaow et al., 2000).

Only relatively few of the many isoflavones identified in plants possess estrogenic activity. Examples of such substances include daidzein and genistein, their glucosides daidzin and genistin, and their methyl ether derivatives formononetin and biochanin A, respectively (Price and Fenwick, 1985). Similarly, only a few of the plant coumestans exhibit estrogenic activity, the most important of which are coumestrol and 4'-methoxy-coumestrol (Figure 1.1). Lignans, as the precursors of

TABLE 1.2
Nomenclature of Lignans

Compound	Lignan Class	Nomenclature
Secoisolariciresinol (SDG)	Oxydiarylbutane	([<i>R</i> -(<i>R</i> [*] , <i>R</i> [*])]-2,3-bis[(4-hydroxy-3-methoxyphenyl)methyl]-1,4-butanediol)
Matairesinol	Diarylbutyrolactone	([3 <i>R</i> - <i>trans</i>]-dihydro-3,4-bis[(4-hydroxy-3-methoxyphenyl)methyl]-2(3 <i>H</i>)-furanone)
Lariciresinol	Furanoid	([2 <i>S</i> -(2 α ,3 β ,4 β)]-tetrahydro-20(4-hydroxy-3-methoxyphenyl)-4-[(4-hydroxy-3-methoxyphenyl)methyl]-3-furanmethanol)
Isolariciresinol	Tetrahydronaphthalene	([1 <i>S</i> -(1 α ,2 β ,3 α)]-1,2,3,4-tetrahydro-7-hydroxy-1-(4-hydroxy-3-methoxy-phenyl)-6-methoxy-2,3-naphthalenedimethanol)
Arctigenin	Diarylbutyrolactone	((3 <i>S</i> ,4 <i>S</i>)-3-[(3-methoxy-4-hydroxyphenyl)methyl]-4-[(3,4-dimethoxyphenyl)methyl]dihydro-2(3 <i>H</i>)-furanone)
Pinoresinol	Furofuran	([1 <i>S</i> -(1 α ,3 α ,4 β ,6 α)]-4,4'-(tetrahydro-1 <i>H</i> ,3 <i>H</i> -furo(3,4- <i>f</i>)furan-1,4-diyl)bis(2-methoxyphenol))
Nordihydroguaiaretic acid		(1,4-bis[3,4-dihydroxyphenyl]-2,3-dimethylbutane) (NDGA)
Hinokinin	Diarylbutyrolactone	(3 <i>S</i> ,4 <i>S</i> ,3,4-bis(1,3-benzodioxol-5-ylmethyl)dihydro-2-(3 <i>H</i>)-furanone)

lignan formation in cell walls, are present as minor constituents in many plants. They contain a 2,3-dibenzylbutane structure (Table 1.2) and were first identified in humans in the late 1970s following studies in monkeys in which compounds were detected in the urine with apparent similarities to urinary steroid hormone metabolites (Setchell and Adlercreutz, 1988).

In plants, isoflavones play major roles in the defense response to pathogen attack (Rivera-Vargas, Schmitthenner, and Granham, 1993) and in nodulation and nitrogen fixation (Pueppke, 1996). Isoflavones also impart negative taste compounds to foods (Okubo et al., 1992), and the reduction of isoflavone concentrations would be of value for other products. A benefit of manipulating isoflavone concentrations is that increased levels of isoflavones may increase resistance to various pathogens (Padmavati and Reddy, 1999). Developing other grain crops that can synthesize isoflavone would provide food manufacturers with alternatives to soy for use in their products.

1.2.2 CHEMISTRY

The basic structural feature of isoflavonoids is two benzene rings linked through a heterocyclic pyrane ring at the 3-position. The most abundant isoflavonoids are genistein and daidzein. Other compounds in this group include formononetin and biochanin A, which in humans can be metabolized to daidzein and genistein, respectively. Daidzein

TABLE 1.3

Nomenclature, $[M + H]^+$, Fragment Ions, UV $\lambda_{\max}$, and Structural Characteristics of Isoflavones

Compound	[M + H] ⁺ (<i>m/z</i>)	Fragment Ion (<i>m/z</i>)	$\lambda_{\max}^b$ (nm)	Isoflavone Part						
				R ₁	R ₂	R ₃	R ₄	R ₅	MW	
Daidzin (daidzein-7- <i>O</i> -β-D-glucoside)	417	255	248, 302sh	H	H	H	glc	H	416	
Glycitin (glycitein-7- <i>O</i> -β-D-glucoside)	447	285	257, 320	H	H	OMe	glc	H	446	
Genistin (genistein-7- <i>O</i> -β-D-glucoside)	433	271	258, 330sh	H	OH	H	H	H	270	
Glycitein	285		257, 320	H	H	OMe	H	H	284	
Daidzein	255		248, 302	H	H	H	H	H	254	
Genistein	271		260, 330sh	H	OH	H	H	H	270	
Formononetin	269		250, 301sh	Me	H	H	H	H	268	
Biochanin A	285		262, 325sh	Me	OH	H	H	H	284	

can be further converted to *O*-desmethylangolensin and equol, and genistein can be converted to *p*-ethyl phenol. Lignans are a group of diphenolic compounds with dibenzylbutane skeleton structures. The mammalian lignans enterolactone and enterodiol are produced by the bacterial flora in the colon from the plant lignans matairesinol and secoisolariciresinol, respectively (Kardinaal, Waalkens-Berendsen, and Arts, 1997).

1.2.2.1 Ultraviolet Absorption Spectrum

Isoflavonoids have distinct ultraviolet (UV) absorption spectra (Table 1.3); it is therefore possible to enhance detection selectivity using detector wavelengths at 260 and 280 nm, as well as to provide a positive confirmation of identity. The spectrum of isoflavones consists of two absorption maxima in the ranges 245 to 275 and 300 to 330 nm, with low relative intensities in the latter range. The UV absorption spectra of daidzein, formononetin, and genistein in methanol ($\lambda_{\max}$) are 238sh, 249, 260sh, 303sh; 240sh, 248, 259sh, 311; and 261, 328sh nm, respectively (Markham, 1982). The concentration of stock solutions of isoflavonoids can be determined by absorbance readings at the wavelength with maximum absorption ($\lambda_{\max}$) using molar extinction coefficients (ϵ) after proper dilution with ethanol (acetonitrile for coumestrol) using the following values: daidzein ($\lambda_{\max} = 250$ nm, $\epsilon = 20,893$), genistein ($\lambda_{\max} = 263$ nm, $\epsilon = 37,154$), formononetin ($\lambda_{\max} = 256$ nm, $\epsilon = 29,512$), biochanin A ($\lambda_{\max} = 263$ nm, $\epsilon = 27,542$), coumestrol ($\lambda_{\max} = 339$, $\epsilon = 22,330$), and equol ($\lambda_{\max} = 281$ nm, $\epsilon = 6761$) (Franke et al., 1994). Therefore, monitoring carried out at 260, 280, and 342 nm (for coumestrol) provides sensitive detection at or very

near the absorption maximum of isoflavonoids (Figure 1.2) (Franke et al., 1995). Absorption spectra of the isoflavones daidzein, genistein, formononetin, and biochanin A in water show strong absorption between 250 and 270 nm and a rather weak band or shoulder in the 300- to 350-nm region. Coumestrol has maxima at 240 and 355 nm (Beekman et al., 1999). In electrospray mass spectrometry, positive ionization of genistein yields two prominent ions at $m/z = 271$ and 312 at a ratio of 4.5:1. These correspond to the singly protonated ion of genistein, $[MH]^+$, and the commonly observed acetonitrile solvent adduct $[M:CH_3CN:H]^+$. With daidzein, a single prominent ion is observed at $m/z = 255$, corresponding to $[MH]^+$ (Jung et al., 2000). Infrared (IR) spectral characteristics of malonylated soybean isoflavones 6''-O-malonyldaidzin, 6''-O-malonyglycitin, and 6''-O-malonylgenistin from soybean seeds are similar (1735 and 1695 cm^{-1}) (Kudou et al., 1991).

1.2.2.2 Fluorescence Spectrum

The fluorescence properties of the isoflavones daidzein, genistein, formononetin, biochanin A, and coumestrol are markedly different despite the closely analogous molecular structures. Under aqueous alkaline conditions, daidzein, formononetin, and coumestrol exhibit strong fluorescence, whereas isoflavones with an additional hydroxy group at the 5-position, genistein and biochanin A, do not show any emission at all. Both daidzein and formononetin show a broad band without vibrational details, with a maximum at 478 nm due to their molecular structures. The spectrum of coumestrol emerges at distinctly shorter wavelengths with a maximum at 441 nm, although a broad band is also observed (Beekman et al., 1999).

1.2.3 BIOSYNTHESIS

1.2.3.1 Isoflavonoids

The biosynthesis of isoflavonoids has recently been reviewed by Dixon (1999). Isoflavones may be either constitutively synthesized under the control of developmental programs or induced in response to environmental stress such as pathogen infection. Isoflavones are synthesized by a branch of the phenylpropanoid pathway of secondary metabolism by specific enzymes, L-phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate ligase (4CL), as well as the acetyl-CoA carboxylase (ACCase) (Dixon and Pavia, 1995) (Figure 1.3). The B-ring of the C_{15} of isoflavonoids originates from the phenylpropane unit of 4-coumaroyl-CoA whereas the A-ring is derived from acetyl-CoA via the condensation of malonyl-CoA (Dixon, 1999). The isoflavone synthase catalyzes the first step of isoflavone biosynthesis, the oxidation of 7,4'-dihydroxyflavanone (liquiritigenin) or 5,7,4'-trihydroxyflavanone (naringenin) to daidzein or genistein, respectively. Conversion of naringenin to genistein is carried out via a protein with isoflavone synthase activity. The presence of isoflavone synthases in other legumes and sugarbeet and the potential for producing isoflavones in non-isoflavone-producing crops have been demonstrated (Jung et al., 2000).

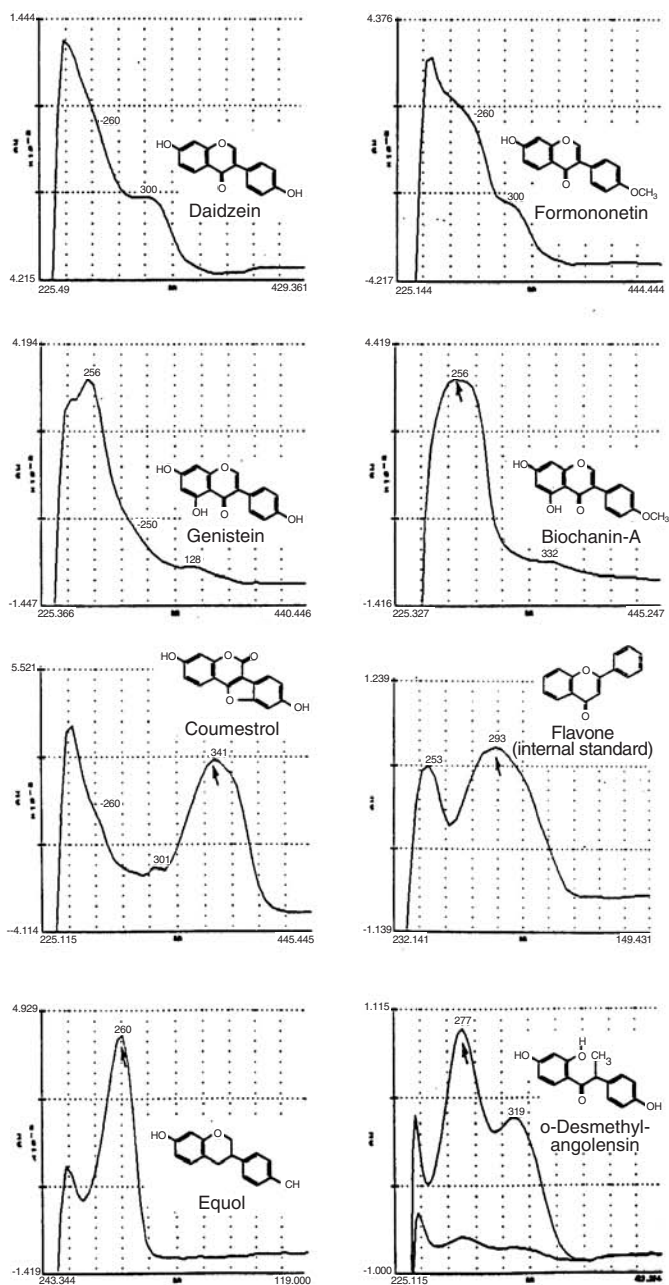


FIGURE 1.2 Structure and UV scan of phytoestrogens. (Reprinted with permission from Franke, A. A., et al., *Proc. Soc. Exp. Biol. Med.*, 208, 18–26, 1995.)

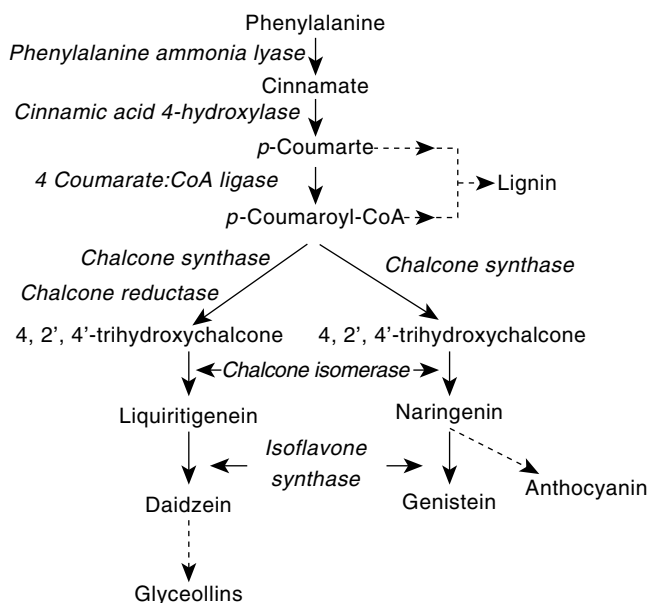


FIGURE 1.3 Isoflavonoid biosynthesis via the phenylpropanoid pathway. (Reprinted with permission from Jung, W., et al., *Nat. Biotechnol.*, 18, 208–212, 2000.)

1.2.3.2 Lignan

The biosynthesis of lignan has recently been revised based on the discovery of the dirigent proteins that guide phenolic radical coupling (Davin et al., 1997; Davin and Lewis, 2000). Lignans are derived mainly via differential partitioning of the mono-lignol, coniferyl alcohol to yield the lignan (+)-pinoresinol, which in turn serves as the precursor of both (–)-secoisolariciresinol and (–)-matairesinol (Figure 1.4). Two genes encoding the corresponding protein involved in the formation of pinoresinol and lariciresinol have been obtained from developing flaxseed (Ford et al., 2000). Elucidation of the pathway leading to the cancer chemopreventive agent (–)-secoisolariciresinol (SDG) was first accomplished using *Forsythia intermedia*, a plant that both produces and further metabolizes enantiometrically pure (+)-pinoresinol, a dimeric lignan formed from (*E*)-coniferyl alcohol. It involves the regio- and stereoselective intermolecular phenoxy radical coupling of two molecules of (*E*)-coniferyl alcohol by a pinoresinol dirigent protein to yield pinoresinol. Sequential enantiospecific reduction of this intermediate then occurs by a reductase to consequently generate lariciresinol and then SDG. Glycosilation of SDG is accomplished by secoisclariresinol diglucosyl transferase that appears to be localized mainly in the seed coat. Elucidation of the lignan biosynthetic pathway leads to the development of strategies for enhancing the levels of SDG in staple dietary foodstuffs and maximizing yields of lignans used to treat or protect against human disease (Jung et al., 2000).

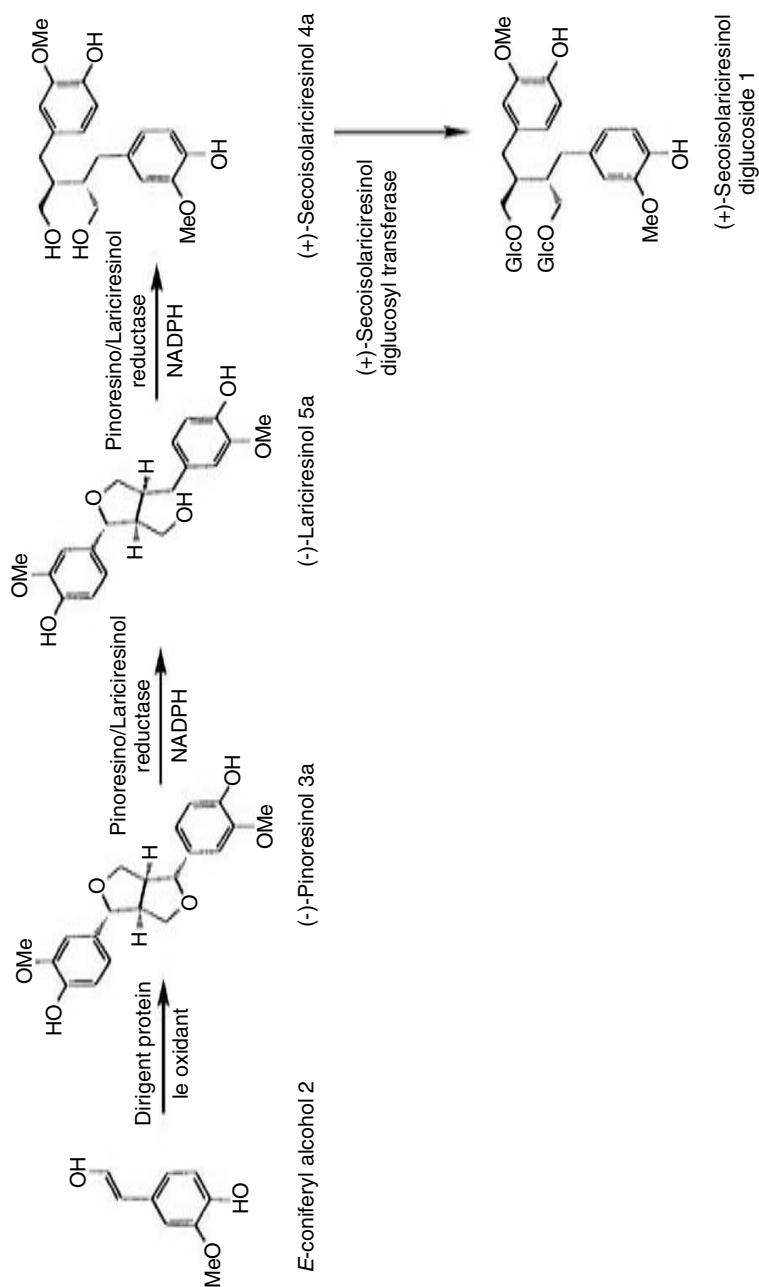


FIGURE 1.4 Biosynthesis of the lignan secoisolariciresinol diglucoside (SDG) in flaxseed. (From Ford, T. D., Davin, L. B., and Lewis, N. G., Proceedings of the 58th Flax Institute of the United States, Fargo, ND, 2000. With permission.)

1.2.4 METABOLISM

The common dietary phytoestrogens, isoflavones and lignans, show similar patterns of metabolism in animals and humans. Both isoflavones and lignans undergo significant metabolism by bacteria in the gastrointestinal tract (Setchell and Adlercreutz, 1988). In foods the isoflavones are conjugated with a glucose to form glycosides, and in soybean the major glycosides are daidzin, genistin, and glycitin. These glycosides are deconjugated to their aglycones daidzein, genistein, and glycitein by acid hydrolysis or bacterial action in the rumen of cows and sheep. Daidzein and genistein are further converted to equol and *p*-ethylphenol in the rumen of sheep (Cox and Davies, 1988). In humans, daidzein and genistein are metabolized to dihydrodaidzein and dihydrogenistein, respectively, which are further metabolized to equol and *O*-desmethylanlangenolins (*O*-DMA) and 6'-hydroxy-*O*-DMA in the case of genistein (Setchell and Adlercreutz, 1988).

The lignans also occur in plants as SDG and matairesinol with glucose residues in the diglucosides attached to the phenolic or side chain OH groups. The glucose and methyl groups are removed by colonic bacterial flora during fermentation to form the diphenols enterodiols which are further oxidized to enterolactone. Matairesinol is converted to enterolactone through dehydroxylation and demethylation (Kurzer and Xu, 1997).

Absorption of the dietary phytoestrogens is facilitated by hydrolysis of the sugar moiety by human gut bacterial β -glucosidases and gastric hydrochloric acid and by β -glucosidases in foods (Kelly et al., 1993). After absorption in the small intestine, isoflavones and lignans are conjugated with glucuronic acid and sulfate, and the conjugates excreted through urine and bile and undergo enterohepatic circulation. The conjugated phytoestrogens, after excretion in the bile, are deconjugated once again by gut bacteria (Kurzer and Xu, 1997). As dietary phytoestrogen metabolism is predominantly determined by the gastrointestinal flora, antibiotic use or bowel disease and gender will modify metabolism. Concurrent dietary intake, in particular high dietary fiber, vegetable and fruit intake, and duration of exposure, exert a major influence on lignan and isoflavone metabolism (Murkies, Wilcox, and Davis, 1998).

1.3 METHODS OF ANALYSIS

1.3.1 SAMPLE PREPARATION AND EXTRACTION

1.3.1.1 Sample Preparation

Sample preparation and extraction are important in the analysis of phytoestrogens, since large losses can occur depending on the technique used and the isoflavone or lignan investigated (Bingham et al., 1998). Prior to extraction, samples are sometimes spiked with various volumes of stock solution containing 20% of standard daidzein, genistein, and biochanin A (Hutabarat, Mulholland, and Greenfield, 1998). The spiking solution (1.25% w/v *tert*-butylhydroquinone in methanol) can also be combined with the extractant (1 *M* HCl) (Wang et al., 1990). In the case of serum, sodium acetate buffer (0.1 *M*, pH 5.0) and an interval standard containing d₄-equol, d₄-daidzein, d₄-genistein, d₄-enterodiol, and d₄-enterolactone are added and equilibrated

for 1 h at room temperature prior to hydrolysis that is allowed to proceed overnight at 37°C after the addition of β -glucuronidase laryl sulfatase (Morton et al., 1999). The choice of flavone as the internal standard is based on its excellent similarity with phytoestrogens, its elution in an “empty” and “late” part of the chromatogram avoiding interference with the analytes, and its stability against heat and acids (Franke et al., 1995). Internal standards such as *p*-nitrophenol (30 mg/l) have also been used with solvents (66% acetonitrile, 0.4% NaCl in water) during extraction (Vänttinen and Moravcova, 1999). The use of internal standards is infrequent, although it allows for the precise determination of analyte recoveries and can account for losses during the extraction process. Thus, only 7 out of 22 studies on phytoestrogens content of foods used an internal standard (Reinli and Block, 1996). As a result, there is very little available information on isoflavones and lignan precursors in foods, and most investigators have relied on urine and blood levels as markers of intake (Bingham et al., 1998).

1.3.1.2 Extraction

Extraction methods vary widely depending on the sample and the phytoestrogen of interest. Several different solvents are in use, including ethanol, methanol, acetonitrile, diethyl ether, and acetone for extraction, although ethanol and methanol appear to be the most common. Samples are extracted with 80% methanol (1:6 meal to methanol ratio) for 30 min, with recovery rates for genistein and daidzein >90% with intraday and interday coefficients of variance below 2% (Wang et al., 2000a); aqueous methanol at elevated temperature (80%, 60°C for 2 h with occasional shaking) (Morton et al., 1999); methanol–water (3:1, v/v, reflux for 1 h under nitrogen) (Rong et al., 1998); acidified ethanol (2 *M* HCl containing 0.05% BHT and 20 ppm flavone and refluxing at 80°C for 1 h) (Hutabarat, Mulholland, and Greenfield, 1998); acidified methanol (1 *M* HCl with gentle stirring over a steam bath for 2 h) (Wang et al., 1990); aqueous ethanol (80%, refluxing for 2 h) (Setchell, Welsh, and Lim, 1987); acidified ethanol (3 *M* trifluoroacetic acid [TFA] in 96% ethanol, refluxing at 100°C for 1 h) (Beekman et al., 1999); and acetonitrile (66% with 0.4% NaCl and 30 mg/l *p*-nitrophenol as an internal standard in water for 1 h) (Vänttinen and Moravcova, 1999). The best extraction efficiencies of soy phytoestrogens are obtained by refluxing 2.3 h with 77% ethanol containing 2 *M* HCl (Franke et al., 1995). Reversed-phase high-performance liquid chromatography (HPLC) analysis show that in most foods the β -glucosides predominate when isoflavones are extracted with hot aqueous methanol or ethanol (80°C, 4 h). Extraction at room temperature results in a different chromatogram with a predominance of 6'-*O*-malonylglucoside (Barnes et al., 1998). Lipid removal by hexane partitioning results in a concentration-dependent loss of isoflavone upon washing with hexane; therefore, lipid removal is deemed not essential for isoflavone quantitation. Omission of the lipid removal step also reduces the sample preparation time substantially. A simple method to extract isoflavones from an aqueous solution is to partition it with an organic solvent immiscible in water; ethyl acetate is safer to handle and more efficient than diethyl ether commonly used for this purpose. The recoveries of the internal standards for this method are in range of 70 to 90% within these limitations, and

TABLE 1.4
Effect of Hydrolysis on the Concentration of Isoflavones
in Soy Food

	Genistein [mg kg ⁻¹ DW]			Daidzein [mg kg ⁻¹ DW]		
	Average	SD	RSD (%)	Average	SD	RSD (%)
Soy flour ^a	53.0	4.9	9.33	68.0	8.0	11.76
Soy flour ^b	406.1	58.9	14.51	516.1	80.9	15.68
Tofu ^a	76.4	2.3	3.03	74.0	1.6	2.14
Tofu ^b	726.9	38.4	5.28	644.5	2.4	0.37

Note: DW – dry weight; SD – standard deviation; RSD – relative standard deviation.

^a Without hydrolysis.

^b With enzymatic (β -glucuronidase) hydrolysis.

Source: From Vääntinen, K. and Moravcova, J., *Czech. J. Food Sci.*, 17, 61–67, 1999.
 With permission.

the method is accurate to at least 100 ng of daidzein or genistein per gram of freeze-dried food (Liggins et al., 1998). Isoflavones from food matrixes (e.g., meat products) are extracted using *N,N*-dimethylformamide (DMF)–water (2:1, v/v) or with acetonitrile–water (2:1, v/v). The acetonitrile–water solvent system has the advantage of extracting low levels of fat that can interfere with the measurements by affecting the electroosmotic flow (Mellenthin and Galensa, 1999).

1.3.1.3 Hydrolysis

Since phytoestrogens exist in plants mainly as glycoside conjugates and in biological fluids from humans and animals as glucuronide or sulfate conjugates, hydrolysis of the conjugate moiety is required prior to HPLC analysis. The hydrolysis step should be considered essential when analyzing animal tissues or fluids, but unnecessary for soy protein preparations (Setchell, Welsh, and Lim, 1987). Although most of the analytes are heat and acid stable, enzymatic rather than acidic hydrolysis for urine analysis is recommended in order to include the metabolite equol in the assay which degrades under acidic conditions (Franke et al., 1995). Enzymatic hydrolysis is also more effective than acid hydrolysis, especially if the latter is performed at elevated temperatures leading to partial destruction of isoflavones (Table 1.4). Hydrolysis of the glycosidic bond is best accomplished overnight at 37°C with enzyme using β -glucosidase preparation in 0.1 *M* acetate buffer (pH 5.0) (Setchell, Welsh, and Lim, 1987), in borate buffer pH 4.0 (Vääntinen and Moravcova, 1999), or a preparation of *Aspergillus niger* since it produces good consistent recoveries from different isoflavone glycoside sources (Liggins et al., 1998). Crude preparations of *Helix pomatia*, β -glucosidase from almonds, and acid hydrolysis produce reasonable recoveries of isoflavones from soy flour, but are not applicable to different sources of isoflavone glycosides (Liggins et al., 1998). A second hydrolysis may be performed with a combined β -glucuronidase and sulfatase preparation in 0.5 *M* acetate buffer (pH 4.5), and extraction and recovery of

the isoflavones can be repeated (Setchell, Welsh, and Lim, 1987). For acid hydrolysis, the sample is incubated in TFA at 95°C for 1 h. After incubation, the mixture is neutralized with CaO and then filtered prior to high-performance capillary electrophoresis analysis (Vänttinen and Moravcova, 1999).

1.3.1.4 Lignan Extraction

Lignan extraction with aqueous alcohol is superior to other solvents and results in the extraction of an ester-linked complex that can be chromatographed on reverse-phase HPLC columns, although quantitation is not possible. SDG is generally isolated by treating an aqueous alcoholic extract with sodium methoxide (1.6% NaMeOH in anhydrous MeOH). The resulting extract is acidified (4 N H₂SO₄ to pH 3.0) prior to purification on silica gel prepared in CHCl₃ and eluted with CHCl₃-MeOH-H₂O (65:35:50) to obtain SDG. HPLC analysis of SDG is possible, although several other contaminating compounds elute very close to SDG. An alternative to the isolation of the ester complex first is the enzymatic conversion of the lignan glycosides to the corresponding aglycone reported by Obermeyer et al. (1995). Although β -glucuronidase releases the aglycone SDG from the complex, hydrolysis is incomplete. Apparently base hydrolysis produces the highest yield of SDG due to complete hydrolysis (Westcott, Muir, and Northrup, 1998). Combinations of enzymatic and acid hydrolysis have been proposed to release the aglycones that can be derivatized and analyzed by gas chromatography (GC). However, an artifact, anhydrosecoisolariciresinol, is produced as a result of acid hydrolysis, thereby producing an unreliable estimate of the lignan content of flax and its products. This artifact is now considered as a naturally occurring lignan, chemically identical to shonanin, and as such should be quantified alongside the other phytoestrogens (Liggins, Grimwood, and Bingham, 2000). The aqueous base hydrolysis is robust and produces SDG as the principal lignans with cinnamic acid glycosides in trace amounts. The glycosides can then be quantitatively converted to aglycones that are analyzed by HPLC or derivatized and analyzed by GC (Muir et al., 2000).

1.3.1.5 Hydrolysis of Glycosidic Bonds of Lignans

Two basic approaches have been used to hydrolyze the glycosidic bonds between plant lignans and carbohydrates, and both methods have been used to produce analytical results presenting the lignan concentrations in various foods (Thompson et al., 1991; Mazur et al., 1996). The Thompson method employs anaerobic *in vitro* fermentation of food containing plant lignans with gut bacteria producing enterolactone and enterodiols, the concentration of which is subsequently measured by GC and flame ionization detection. The second method described by Mazur et al. (1996) employs hot acid to break the glycoside bond and is a multistep process using both enzyme and hot acid to hydrolytically remove the carbohydrate component of lignan and isoflavone phytoestrogens in food. In this procedure, the food extract is first subjected to enzyme hydrolysis, liberating the isoflavones from their respective glycosides.

Since the lignans are not completely hydrolyzed by enzymes, hot acid is used to liberate the aglycones that are partitioned off with organic solvents. Both organic fractions of isoflavone and lignan aglycones are then combined, further purified,

derivatized, and analyzed by gas chromatography-mass spectrometry (GC-MS). However, the stability of products from acid hydrolysis has recently been questioned (Liggins et al., 1998). It appears that a naturally occurring lignan called shonanin (3,4-divanillyltetrahydrofuran) is also liberated from its glycosides alongside SDG as a result of acid hydrolysis. In order to account for the presence of shonanin and its hydrolytic product enterofuran in foods, Liggins, Greenwood, and Bingham (2000) modified the procedure of Mazur et al. (1996) and simplified it for the quantification of lignans, SDG, matairesinol, and shonanin in food after hydrolytic removal of any conjugated carbohydrate. The modification includes acid hydrolysis of food samples for 1, 2, and 3 h, followed by neutralization with sodium hydroxide, separation of the aglycones from aqueous to organic phase, and quantitation of the aglycones by GC-MS after the formation of trimethylsilyl derivatives of the lignans. The content of lignans in reference foods is reported as the combined concentrations of SDG and shonanin, alongside the concentration of matairesinol after 1, 2, and 3 h of hydrolysis, since optimum time of hydrolysis for the maximum yield of lignans varies between foodstuffs.

1.3.2 SOLID-PHASE EXTRACTION

Klejdus, Vitamvasova, and Kuban (1999) used different solid-phase extraction (SPE) methods for isolation of daidzein, genistein, formononetin, and biochanin A to compare two red clover varieties. Filtered ethanolic (80%) extract of isoflavone is diluted with water (1:3, extract to water) prior to SPE extraction. The cartridges are conditioned with either water or methanol. Samples are then passed through and washed with methanol (5 or 10%) to remove impurities, and the retained isoflavones are eluted with 80% methanol. The extract is evaporated to dryness, dissolved in the mobile phase, and directly injected onto a reversed-phase HPLC column.

C₁₈ and C₈ sorbents have low recoveries due to the extremely hydrophobic surfaces. The best recoveries $\approx 100\%$ are obtained with conditioning on an Spe-ed ABN cartridge (among the cartridges tested C₁₈, C₈, Amide 2, RP 105, ABN, and HLB). Conditioning is not a prerequisite since high recoveries of over 90% are obtained with ABN cartridges. SPE provides for high reproducibility [low relative standard deviation (RSD)] and low consumption of plant materials. SPE with polyamide or alternatively RP₁₈ cartridges is required for samples with low isoflavone content or with a matrix interfering with the experiment. The RP₁₈ columns should be conditioned with two column volumes of methanol followed by the same volume of water prior to eluting the polyphenols with methanol. Only two column volumes of water are required to pretreat the polyamide column (Mellenthin and Galensa, 1999).

1.3.3 CHROMATOGRAPHIC SEPARATION

1.3.3.1 Paper Chromatography

Two-dimensional paper chromatography has been used to demonstrate the presence of the phytoestrogen coumestrol in alfalfa (Bickoff et al., 1967). This method was later used by Knuckles, de Fremery, and Kohler (1976) to ascertain the coumestrol content of vegetable products. According to this method, coumestrol is extracted by

soaking the products in 75% ethanol for 16 h. The extract is applied on the chromatogram that is first developed in acetic acid–water (1:1, v/v), then in isopropyl alcohol–ammonium hydroxide (2:1, v/v). The intensity of the coumestrol spots is measured fluorometrically without elution from the paper. The detection limit of coumestrol is 0.1 µg/g. Thirteen of the sixteen vegetable products analyzed by two-dimensional paper chromatography contained coumestrol (Knuckles, de Fremery, and Kohler, 1976).

1.3.3.2 Thin-Layer Chromatography

Thin-layer chromatography (TLC) has been used in the identification of isoflavonoids. Precoated polyamide 6 TLC plates have been used for the initial fractionation of isoflavones and phenolics from soybeans and soy products (Pratt and Birac, 1979). The methanolic extracts are spotted on 20 × 20 cm plates and developed using methanol–acetic acid–water (90:5:5). Bands are eluted with methanol and rechromatographed on polyamide using chloroform–methanol–methyl ethyl ketone (12:2:1). For aglycones, the plates are developed in ethyl acetone–petroleum ether (3:1) and then in ethanol–chloroform (1:1). Bands are detected with appropriate chromogenic sprays or with a UV lamp at 366 nm (Pratt and Birac, 1979). Diethyl ether extract chromatographed on a silica plate developed three times in dichloromethane–methanol (95:5, v/v) gave the following R_f : daidzein, 0.20; genistein, 0.30; formononetin, 0.55; and biochanin A, 0.75, respectively (Table 1.5) (Lapcik et al., 1998). TLC carried out on a Kieselgel 60 F₂₅₄ plate using chloroform–methanol–2% acetic acid (7:3:1, v/v lower layer) and visualized by heating at 120°C for 10 min after spraying with 10% H₂SO₄ gave the following R_f values of 0.41, 0.45, 0.43, 0.08, 0.12, 0.10, 0.56, 0.61, and 0.59 for daidzin, glycitin, genistin, 6''-O-malonyldaidzin, 6''-O-malonylglycitin, 6''-O-malonylgenistin, 6''-O-acetyldaidzin, 6''-O-acetylglycitin, and 6''-O-acetylgenistin, respectively (Kudou et al., 1991). Isoflavones obtained by supercritical fluid extraction methods have also been evaluated by TLC on glass plates precoated with silica gel with a fluorescent indicator (Chandra and Nair, 1996). The supercritical carbon dioxide extracts analyzed using chloroform–methanol (10:1) produced the following R_f values of 2.5, 3.1, 5.0, and 5.7 for daidzein, genistein, formononetin, and biochanin A, respectively, when visualized under a UV lamp at 254 nm (Chandra and Nair, 1996).

TLC-silica (straight phase) and rectromatography on reversed-phase octadecyl-silica column HPLC has been used to fractionate isoflavonoids from diethyl ether extracts of beer (Lapcik et al., 1998). The fractions are analyzed by radioimmunoassay (RIA) specific for daidzein/formononetin and genistein/biochanin A. The diethyl ether extract is chromatographed on a Merck PSC silica plate along with standards of daidzein, formononetin, genistein, and biochanin A in separate tracks. The plate is developed three times in dichloromethane–methanol (45:5, v/v), viewed under UV light, scraped, eluted with methanol, and analyzed by RIA. The fractions containing isoflavonoids are rechromatographed on reversed-phase HPLC, eluted, collected, and assayed for immunoreactivity with two antisera (daidzein/formononetin and genistein/biochanin A) (Lapcik et al., 1998). The presence of lignans and their precursors in flaxseed has been modified by TLC and quantified by an HPLC method

TABLE 1.5
Chromatographic Properties of Isoflavonoids

	TLC System 1 ^a R _f	TLC System 2 ^b R _f	HPLC System 1 ^c Retention Time (min)
Daidzein	0.20	0.29	6.5
Genistein	0.30	0.44	8.5
Formononetin	0.55	0.69	12.8
Biochanin A	0.75	0.81	21.7

Note: R_f, the chromatographic mobility as the ratio of the distance of the center of the spot from the start to the distance between the start and the solvent front.

^a Merck PSC silica plate (Art. 13794) developed three times in dichloromethane–methanol (95:5, v/v).

^b Merck DC-Alufolien (Art. 5583) developed twice in dichloromethane–methanol (95:5, v/v).

^c Column ET 250/4 Nucleosil 100-5 C₁₈; mobile phase, methanol–water (60:40, v/v); flow rate, 0.8 ml/min; temperature, 35°C.

Source: From Lapcik, O. et al., *Steroids*, 63, 14–20, 1998. With permission.

developed by Harris et al. (1994). TLC carried out on an HP Kieselgel 60 F₂₅₄ plate using chloroform–methanol–acetic acid (90:10:1, v/v/v) gave the following R_f values of 0.01, 0.17, 0.57, 0.59, 0.70, and 0.71 for chlorogenic acid, gallic acid, 4-hydroxybenzoic acid, coumaric acid, ferulic acid, and SDG diglucoside, respectively. Due to the success of the TLC and HPLC methods, Harris et al. (1994) suggest that high-performance TLC (HPTLC) may allow for the analysis of SDG and its metabolites in a large number of flaxseed samples in a short time. One of the major advantages of HPTLC is the potential of analyzing samples, especially oilseeds, without employing a defatting step.

1.3.3.3 Gas Chromatography

Isoflavonoids and lignans are normal constituents of body fluids and have been identified in most animal and human body fluids by GC-MS (Setchell and Adlercreutz, 1988). GC-MS has been successfully used in the analysis of the phytoestrogens in urine (Adlercreutz et al., 1986, 1991b; Cassidy, Bingham, and Setchell, 1994; Kelly et al., 1993), plasma (Adlercreutz et al., 1993; Joannou et al., 1995; Morton et al., 1994), saliva (Finlay et al., 1991), semen (Dehennin et al., 1982), prostatic fluid (Morton et al., 1997), and feces (Adlercreutz et al., 1995; Kurzer et al., 1995). The presence of at least one hydroxyl group in the structure of common dietary phytoestrogens makes them difficult for direct GC-MS analyses. The hydroxyl group is generally derivatized with *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA)

prior to GC-MS, making the analysis more time consuming (Barnes et al., 1998). However, GC-MS is accepted as the technique sufficiently sensitive for low-level analysis of lignans and isoflavones (Mazur et al., 1996). In a more recent study (Zeleniuch-Jacquotte et al., 1998), isoflavonoid assay by ion-exchange chromatography and GC-MS showed high coefficients of variation ($\leq 10\%$) unlike those of lignans, indicating that it is not well suited to examine the relation of serum isoflavonoid with disease risk. GC-ion mobility, an atmospheric pressure detection system for gas chromatography, has been used to detect the presence of mammalian lignans in urine samples (Atkinson, Hill, and Shultz, 1993).

Morton et al. (1999) describe a simple, robust method employing isotope dilution (ID) GC-MS for the determination of phytoestrogens in biological samples and food matrices. Briefly, samples are hydrolyzed with β -glucuronidase, the aglycones are extracted, and the phytoestrogen fraction is isolated by chromatography on Sephadex LH 20. This fraction is then derivatized for GC-MS by reaction with BSTFA to form trimethylsilyl derivatives. For GC-MS, the column (silica capillary column 10 μ phase thickness) is programmed from 190° to 245° at 49.9°C/min under a helium pressure of 25 kPa. Calibration standards and samples are injected in a BSTFA reagent in a splitless injector maintained at 230°C. The interface and source are operated at 250° and 230°C, respectively. ID-GC-MS is carried out in the selected ion recording (SIR) mode where the intensity of selected ions for each analyte and deuterated internal standard are monitored and expressed as a peak ratio. A calibration curve of peak height ratio against concentration is used to calculate the concentration of the analyte (Morton et al., 1999). The levels of daidzein and genistein produced from soybean hulls, soybean hypocotyls, dehulled soybeans, and other soy-based foods, as well as the levels of phytoestrogens in the sera from Japanese men, have been evaluated by this method.

Isoflavones, together with other flavonoid aglycones, have been separated into well-resolved sharp, and symmetrical peaks by a rapid and sensitive capillary column GC method (Koupai-Abyazani, Creaser, and Stephenson, 1992). The tailing factor or asymmetric ratio of the trimethylsilyl derivatives of flavonoid aglycones are, on the average, 50% greater in packed column GC than in capillary column GC. As a general rule, the retention times of the flavonoid derivatives increase with the number of ether groups, and substitution at different positions of the flavonoid nucleus affects the retention characteristic differently. For example, the retention times for 4,5,7-trihydroxyisoflavone and 4',6,7-trihydroxyisoflavone are 27.59 and 37.07 min, respectively, due to the ether group in position 6 resulting in a longer retention time. When the ether group is added to position 5, as in the case of daidzein to genistein, the differences in retention times are small (25.92 to 27.59 min). The number of hydroxyl groups of the isoflavones (daidzein and genistein, two and three hydroxyl groups, respectively) has very little effect on retention time.

The main phytoestrogens, matairesinol, SDG, anhydrosecoisolariciresinol, biochanin A, genistein, formononetin, daidzein, and coumestrol, have been determined in dry food samples by a very sensitive, but complicated ID-GC-MS selected ion monitoring (SIM) method (Mazur et al., 1996). This is the only method, at present, by which all these lignans and isoflavonoids may be simultaneously determined in foods. These compounds are measured by ID-GC-MS-SIM using synthesized deuterated internal

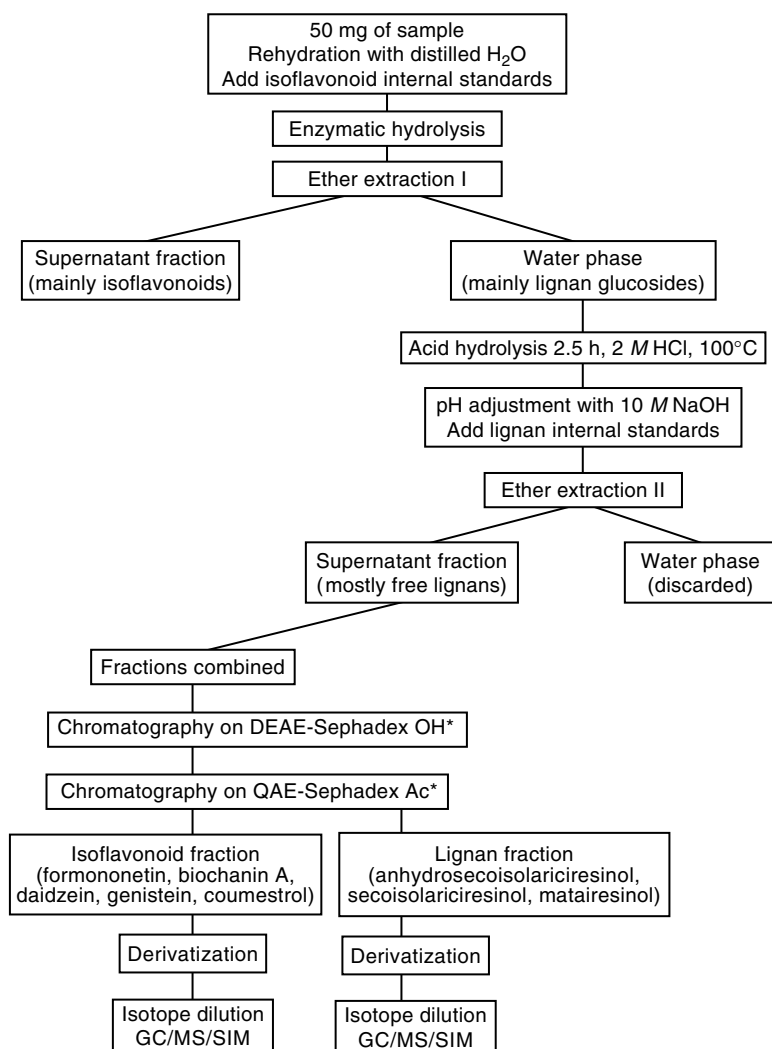


FIGURE 1.5 Flow diagram of the ID-GC-MS-SIM method for the determination of the isoflavonoid and lignan phytoestrogens in food. (From Mazur, W. M. et al., *Br. J. Nutr.*, 79, 37–45, 1998. With permission.)

standards for the correction of losses during the procedure. A significant limitation of lignan analysis by GC-MS resides in the insufficient volatility of the parent compound without derivatization and the conversion of the conjugates to their corresponding aglycones. These limitations can be overcome by devising a series of hydrolysis and purification procedures (Adlercreutz et al., 1993). A description of the main steps of the method is provided in the flow diagram in Figure 1.5. The diphenolic glycosides are converted to their respective aglycones by a three-step hydrolysis involving a rehydration with distilled water, followed by enzymatic and

TABLE 1.6
Relative Retention Times in ID-GC-MS-SIM of
Phytoestrogens and Their Deuterated Homologs

Compound	Time (min)	Relative Retention Time
Phytoestrogen fraction		
Formononetin		0.802
<i>d</i> ₃ -Formononetin		0.800
Biochanin A		0.841
<i>d</i> ₄ -Biochanin A		0.839
Daidzein		1.003
<i>d</i> ₄ -Daidzein ^a	8.821	1.000
Genistein tri-TMS ^b		1.029
di-TMS		1.050
<i>d</i> ₄ -Genistein tri-TMS		1.208
di-TMS		1.049
Coumestrol		1.168
<i>d</i> ₄ -Coumestrol		1.165
Lignan fraction		
ANHSEC		0.916
<i>d</i> ₈ -ANHSEC		0.914
SECO		0.946
<i>d</i> ₆ -SECO		0.945
Matairesinol		1.001
<i>d</i> ₆ -Matairesinol ^a	8.732	1.000

^a Reference compound

^b TMS, trimethylsilyl ether.

Source: From Mazur, W. et al., *Anal. Biochem.*, 233, 169–180, 1996.
 With permission.

acid hydrolysis. Enzymatic hydrolysis is carried out with *H. pomatia* juice which hydrolyzes the isoflavonoid glycosides effectively and the lignan glycosides minimally. Thus, the ether extract contains mainly lignans that are hydrolyzed completely with acid without affecting the isoflavones. Purification and separation are carried out in two ion-exchange chromatographic steps followed by derivatization and GC-MS. Neutral steroids and other contaminants are removed by DEAE- and QAE-Sephadex chromatography prior to separation and identification by GC-MS. The method is highly specific because of the use of deuterated internal standards, the retention time of which is very similar to those of the nondeuterated standards (Table 1.6). The within- and between-assay imprecision values of the method vary from 3.1 to 9.6% and 7.0 to 21.2%, respectively. The mean recovery of authentic standards processed through the whole procedure varies from 95.5 to 105.5%. The method gives four to five times higher SDG values in flaxseed compared to published results (Adlercreutz and Mazur, 1997), probable due to the higher accuracy of the methodology in

the determination of SDG and its hydrolytic product, anhydrosecoisolariciresinol. Although the methodology is complicated, it simultaneously determines eight phytoestrogens even at very low levels. However, one disadvantage is that the concentration of the various compounds are so different that analysis has to be carried out twice, once for the abundant compounds and once for the minor components. The sensitivity of the method is approximately 2 to 3 mg/100 g. The method is suitable for the measurements of isoflavonoids and lignans in as little as 50 mg of foodstuffs. This method has been used to measure phytoestrogens in many foods, including 52 leguminous seeds; teas and coffees (Mazur et al., 1998); vegetables; fruits and berries; and oilseeds, nuts, and beer (Lapcik et al., 1998). The ID-GC-MS in the SIM mode has been used in measuring and reporting quantitative results for plant estrogens in grains and cereals, oilseeds and nuts, berries, fruits, vegetables, leafy vegetables, and beverages (Mazur, 1998).

Liggins et al. (1998) also used GC-MS in selected ion mode to develop a simple analytical method for routine quantification of the isoflavones daidzein and genistein in food using synthetic glucosides daidzein and genistein as internal standards, combined with the food prior to extraction. In this method daidzein and formononetin are liberated from their respective glycosides by hydrolytic enzymes from *A. niger* in aqueous buffer. The aglycone isoflavones are partitioned into ethyl acetate, which is evaporated under nitrogen, derivatized with *N*-tert-(butyldimethylsilyl)-*N*-methyl-trifluoroacetamide (TBDMS), and quantified using GC-MS in selected ion mode. Derivatization of isoflavones with TBDMS improves stability, volatility, and chromatographic resolution of the ethers. The recoveries of the internal standards for this method are in the range of 70 to 90% within these limitations, and the method is accurate to at least 100 ng of daidzein or genistein per gram of freeze-dried food (Liggins et al., 1998).

GC-MS in the positive ion mode has been used to confirm the identity of lignans by retention times and mass spectra of their trimethylsilyl ether (TMS) derivatives after separation and purification by HPLC-DAD (Meagher et al., 1999). Apparently lignans with different structures such as isolariciresinol and pinoresinol are detectable only in the positive ion and total ion modes, but not if SIM is utilized. Although the total ion current profile of lignan is complex, lignan peaks are well resolved on the nonpolar column (Figure 1.6). Isolariciresinol has a shorter retention time on GC than other lignans, although it is unresolved on HPLC due to coeluting sterol peaks (Meagher et al., 1999).

1.3.3.4 High-Performance Liquid Chromatography

Although GC with a mass spectrometric detection method applied to the analysis of phytoestrogens is sensitive, it is also time consuming due to the cumbersome sample preparation steps. Separation by liquid chromatography obviates the need for derivatization. Hence, HPLC has proven to be the method of choice since the 1980s. Generally, phytoestrogens can be separated with mixtures of methanol or acetonitrile and aqueous acids or buffers as modifiers using reversed-phase C₁₈ stationary matrices. In the latest review on the measurement of food flavonoids, Merken and Beecher (2000) point to the diverse and ever-increasing use of HPLC

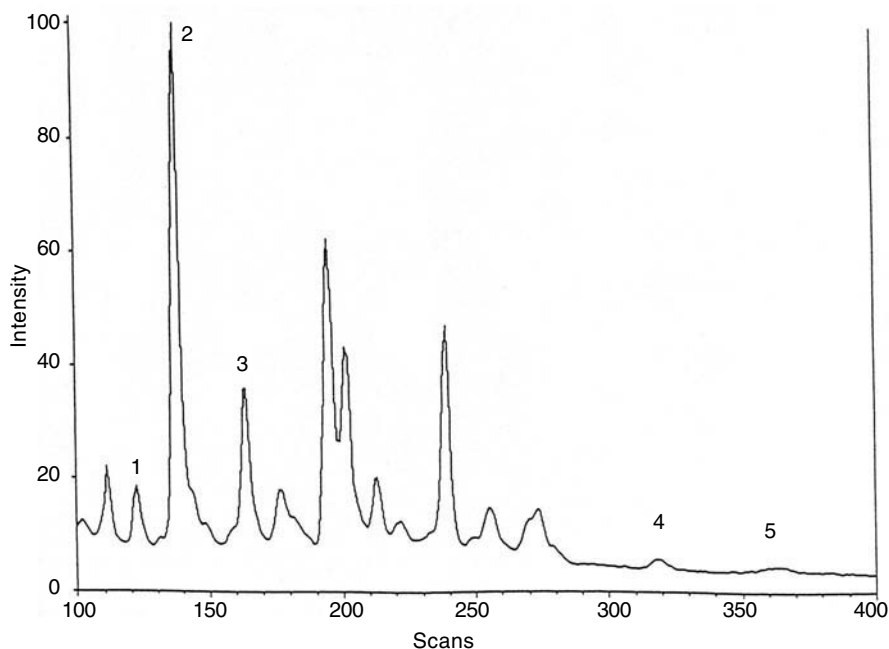


FIGURE 1.6 GC-MS chromatogram of an acid hydrolyzed methanolic flaxseed meal extract. Peaks 1 to 4 are isolariciresinol, SDG, divanillyltetrahydrofuran, and matairesinol, respectively. (Reprinted with permission from Meagher, L. P., et al., *J. Agric. Food Chem.*, 47, 3173–3180, 1999).

in the analysis of isoflavonoids. Several HPLC methods have been tested using various mobile phases and columns (Table 1.7). The most commonly used HPLC method applies a linear gradient for the separation and quantitative analysis of isoflavonoids from soybean and soybean-based products (Barnes, Kirk, and Coward, 1994; Coward et al., 1993; Eldridge, 1982a,b; Ohta et al., 1979; Seo and Morr, 1984; Wang and Murphy, 1994). Nonlinear gradient elution HPLC has also been used for the determination of isoflavonoid and coumestan phytoestrogens from many legumes and legume-based products (Franke et al., 1994, 1995; Murphy, 1981). Most investigators prefer gradient conditions for HPLC to maximize the separation of complex mixtures of phytoestrogens as they exist in plant and food products. Methods for the separation of phytoestrogens in plant extracts by HPLC have been described (Dziedzic and Dick, 1982; Eldridge, 1982a,b; Farmakalidis and Murphy, 1984; Murphy, 1982; Nicollier, 1982; Pettersson and Kiessling, 1984; Sachse, 1984; Seo and Morr, 1984) using gradient elution systems. The gradient HPLC analysis of isoflavones in soybean foods is summarized in Table 1.8. The concentration of acetonitrile increases by 2.25% min, enabling the separation of both the isoflavone β -glucoside conjugates and aglucones in a single chromatographic run (Lee, 2000). Runs are generally 1 h maximum with equilibration between runs. A striking exception is the 340-min run used for the HPLC of isoflavones in soy sauces for pattern recognition analysis (Kinoshita, Ozawa, and Aishima, 1997; Kinoshita et al., 1998).

TABLE 1.7
HPLC of Isoflavones

Sources	Stationary Phase	Mobile Phase ^a	Ref.
American groundnut tubers	C ₁₈ reversed-phase ^b	MeOH, 25–100% ^c	Krishnan, 1998
Infant formula	YMC-Pack ODS-AM 303 (250 × 4.6 mm, S-5 μm 120 Å) ^d	A: 0.1% CH ₃ COOH/H ₂ O; B: CH ₃ COOH/CH ₃ CN ^e	Garrett et al., 1999
Commercial soybean foods	YMC-Pack ODS-AM 303 (250 × 4.6 mm)	A: 0.1% CH ₃ COOH/H ₂ O; B: CH ₃ COOH/CH ₃ CN; gradient: 15–35% B, 0–50 min; 35% B, 50–60 min	Wang and Murphy, 1994
Soy sauces	Wakosil-II 5C ₁₈ HG ^f (250 × 4.6 mm)	A: 0.5 CF ₃ COOH/H ₂ O; B: 0.05% CF ₃ COOH/CH ₃ CN/H ₂ O; gradient: 100% A, 0–20 min; 0–25% B, 20–290 min; 25–50% B, 290–340 min	Kinoshita, Ozawa, and Aishima, 1997; Kinoshita et al., 1998
Soybean foods	Brownless Aquapore C ₈ reversed-phase (300 × 4.5 mm)	A: 0.1% (v/v) CF ₃ COOH/H ₂ O; B: CH ₃ CN; gradient: 0–46.4% B, increasing by 2.25%/min	Coward et al., 1993
Soy foods	Aquapore C ₈ (250 × 4.6 mm)	A: either 0.1% CF ₃ COOH or 2 or 10 mM NH ₄ Oac; B: CH ₃ CN; gradient: 0–50% B, periods ranging from 0–10 to 0–30 min; 100% B for 5 min	Barnes, Kirk, and Coward, 1994
Soy foods	Aquapore C ₈ reversed-phase (100 × 4.6 mm, 300 Å)	A: 10 mM NH ₄ Oac, pH 6.5; B: CH ₃ CN; gradient: 0–50% B, 0–10 min	Barnes et al., 1998b
Soybean cotyledons; soy; soybean seedling tissues	Hibar EC containing Merck Lichrosorb RP ₁₈ ^g 10 μm C ₁₈ reverse-phase packing (250 × 4.6 mm)	A: H ₂ O, pH 3; ^h B: CH ₃ CN; gradient: 0–55% B, 0–25 min; step increase to 100% B, held for 2 min; step return to 100% A	Graham, King, and Graham, 1990; Graham, 1991a,b
Soy products	Reversed-phase Spherisorb 5-μm ODS 2 (250 × 4.6 mm)	CH ₃ CN/H ₂ O to pH 7.5 with Kolthoff's borax-phosphate mixture; A: buffered to 10% CH ₃ CN/H ₂ O; B: buffered to 40% CH ₃ CN/H ₂ O; gradient: 100% A–100% B, 0–30 min; 100% B, 30–50 min	Jones et al., 1989
Soybean	NovaPak C ₁₈ reversed-phase (150 × 3.9 mm, 4 μm)	A: CH ₃ CN; B: 1% CH ₃ COOH/H ₂ O (v/v); isocratic: 33% A, 67% B	Hutabarat, Mulholland, and Greenfield, 1998

TABLE 1.7 (continued)
HPLC of Isoflavones

Sources	Stationary Phase	Mobile Phase ^a	Ref.
Legumes	NovaPak C ₁₈ reversed-phase (150 × 3.9 mm, 4 μm)	A: CH ₃ COOH/H ₂ O (10:90, v/v); B: CH ₃ CN; gradient: 23–70% B, 8–8 min; 23% B, 8–20 min for equilibration	Franke et al., 1994, 1995
Soy foods	NovaPak C ₁₈ reversed-phase (150 × 3.9 mm, 4 μm)	A: CH ₃ COOH/H ₂ O (10:90, v/v); B: MeOH/CH ₃ CN/CH ₂ Cl ₂ (10:5:1, v/v); gradient: 5% B, 0–5 min; 5–45% B, 5–45 min; 45–70% B, 45–51 min; 70–75% B, 51–54 min; 5% B, 54–69 min for equilibration	Franke et al., 1998
Soybean and its processed products	μ-Bondpak C ₁₈ (300 × 3.9 mm, 10 μm) ^j	Isocratic: MeOH/1 mM NH ₄ OAc (6:4)	Wang et al., 1990
Soybean	YMC-Pack ODS-AQ ₂ (250 × 4.6 mm, 5 μm)	A: 0.1% CH ₃ COOH/H ₂ O; B: 0.1% CH ₃ COOH/CH ₃ CN; gradient: 15–31% B in 45 min	Wang et al., 2000a
Soy products	Hypersil C ₁₈ (250 × 4.6 mm, 5 μm)	MeOH/50 mM NaOAc/CH ₃ CN (40:40:20, v/v/v); isocratic 25 min	Müllner and Sontag, 1999
Soy protein	Hypersil C ₁₈ (250 × 4.6 mm, 5 μm)	MeOH/0.1 M NH ₄ OAc (6:4, v/v) pH 4.6 with 0.25 mM/l EDTA; isocratic	Setchell, Welsh, and Lim, 1987
Soy products	Licrosphere RP ₁₈ (250 × 4.6 mm, 5 μm)	A: 20% MeOH/10 mM H ₃ PO ₄ ; B: 100% MeOH; gradient: 0–100% B, 0–55 min	Plewa et al., 1999
<i>Puerarie lobata</i>	Licrosphere RP ₁₈ (250 × 4.6 mm, 5 μm)	A: 1% HCOOH/H ₂ O; B: 5% CH ₃ CN/MeOH; gradient: 15–95% B	Rong et al., 1998

^a All gradients are linear.^b Brand not listed.^c Times not listed.^d Guard column Hichrom RPB (10 × 0.3 mm).^e Gradient not listed, but article refers to Barnes et al. (1994).^f Wakosil-II 5C₁₈ HG (30 × 4.6 mm).^g Merck Lichrosorb RP₁₈ 10 μm C₁₈ reverse-phase packing.^h 1990 paper does not mention pH 3. Adsorbosphere C₁₈ (10 × 4.6 mm, μm).^j Guard column C₁₈/Corasil (37–50 μm).

Isocratic elution systems are also commonly encountered in the analysis of phytoestrogens. Thus, West et al. (1978) used HPLC analysis to separate the isomeric trihydroxyisoflavones and genistein from soybean with isocratic conditions. However, this method was not applicable to phytoestrogens other than genistein. A simple isocratic reversed-phase system, with methanol–0.1 M ammonium acetate, pH 4.6

TABLE 1.8
Gradient HPLC Analysis of Isoflavones in Soybean Products

HPLC Condition

Column: brownlee aquapore C₈ (300 × 4.5 mm ID, 5 μm)

Mobile phase: eluent (A): acetonitrile; eluent (B): water-trifluoroacetic acid (0.1%)

Use gradient of 0–46.4% acetonitrile (2.25% per min) in 0.1% aqueous TFA

Flow rate: 1.5 ml/min

Detection: UV 262 nm

Sample preparation

Extract soybean foods with 80% aqueous MeOH (10 ml/g), containing 1.25 mg fluorescein (internal standard), by stirring 1 h at 60°C and centrifuge (10 min at 2,500 g). Dry the supernatants and redissolve in 50% MeOH (5 ml), defatting by partitioning with hexane (4 × 20 ml). Evaporate the aqueous methanol phase to dryness, suspend in 80% MeOH (10 ml), and centrifuge (2 min at 14,000 g).

Source: From Coward, L. et al., *J. Agric. Food Chem.*, 41, 1961–1967, 1993. With permission.

(60:40, v/v) as the mobile phase on an Hypersil ODS column, has been reported by Setchell, Welsh, and Lim (1987) for the rapid and effective separation of the phytoestrogens daidzein, genistein, coumestrol, formononetin, and biochanin A. In this system phytoestrogens on Hypersil ODS separate and elute in the following order: daidzein, genistein, coumestrol, formononetin, and biochanin A. Methanol is a better modifier than acetonitrile and is essential for the separation of genistein and coumestrol. The pH and buffer concentration of the mobile phase affects the retention, but not the resolution. Increasing the pH and/or the buffer concentration decreases the retention of all compounds while maintaining the resolution (Setchell, Welsh, and Lim, 1987). Hutabarat, Mulholland, and Greenfield (1998) succeeded in obtaining an isocratic separation of all analytes in less than 24 min with acetonitrile (33%) and water–acetic acid (67%) (99:1, v/v). Daidzein elutes at 3 min (capacity factor $k' = 0.92$); genistein elutes at 5 min ($k' = 2.24$); biochanin A elutes at 21 min ($k' = 12.76$), and flavone, an internal standard, elutes at 23 min ($k' = 14.16$). RSD values for six injections of phytoestrogens for this method are 1.26 to 1.51%. The mean recoveries of the compounds ranged from 100 to 127% for daidzein, 101 to 114% for genistein, and 97 to 135% for biochanin A (Hutabarat, Mulholland, and Greenfield, 1998).

Reversed-phase HPLC has frequently been described for the determination of the isoflavones daidzein and genistein (Barbuch et al., 1989; Carlson and Dolphin, 1980; Coward et al., 1996; Franke and Custer, 1994, 1996; Franke et al., 1998; Gamache and Acworth, 1998; Kitada et al., 1986; Lundh, Pettersson, and Kiessling, 1988; Murphy, 1981; Wang et al., 1990). Up to 12 isoflavones, including daidzin, glycitin, genistin, malonyl daidzin, malonyl glycitin, malonyl genistein, daidzein, acetyl genistein, acetyl daidzin, and genistein, can be detected in soybean and soy-derived products (Plewa et al., 1999; Wang et al., 2000a). In the mature soybean, only the glucosides and the malonyl glucosides are found. The acetyl glucosides and the aglycones are degradation products of the malonyl glucosides and glucosides, respectively. These generally appear during commercial processing after acid- or

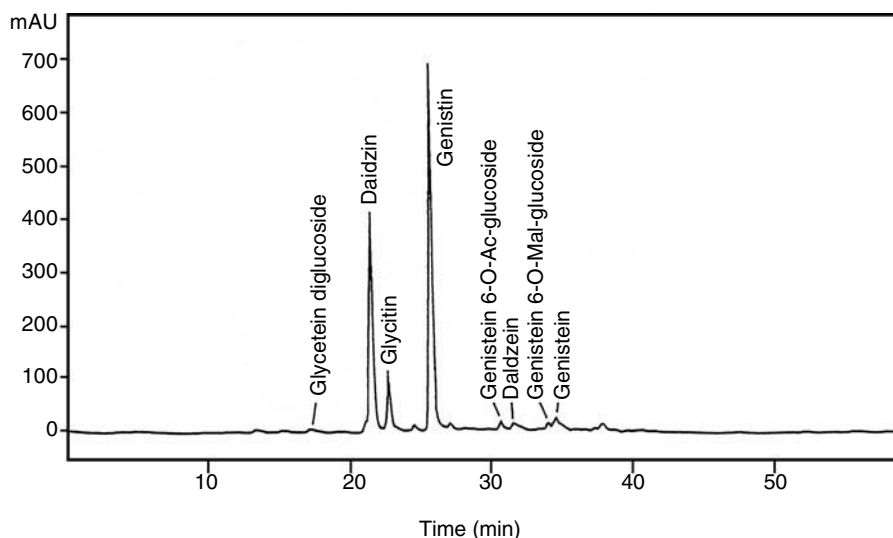


FIGURE 1.7 HPLC chromatogram of isoflavonoids extracted with 70% methanol from a soybean byproduct. (Reprinted with permission from Plewa, M. J., et al., *Teratogenesis Carcinog. Mutagen.*, 19, 121–135, 1999.)

heat-mediated hydrolysis of the malonyl glucosides and glucosides to form acetyl glycosides and the aglycones. The soy isoflavones are separated by HPLC according to their retention times (Figure 1.7, Table 1.9). Generally, the glycosides elute quantitatively in the early part of the HPLC chromatogram (between 10 and 25 min), with aglycones eluting much later (t_R between 34 and 45 min) (Rong et al., 1998).

Franke et al. (1995) describe a simple and fast procedure to extract and simultaneously hydrolyze phytoestrogens and their conjugates from foods and present a fast and selective HPLC method for precise determinations of the most common phytoestrogen genistein, biochanin A, daidzein, formononetin, and coumestrol using flavone as the internal standard. A NovaPak C₁₈ column in combination with an acetonitrile–acetic acid (10% in water) elution system showed the best selectivity, recovery, and peak shape for all analytes of interest among several HPLC columns and solvent system tested using authentic phytoestrogen standards (Figure 1.8). Monitoring at 260, 280, and 342 nm at or very near the absorption maximum of the analytes provided sensitive detection. Coumestrol is additionally monitored with a fluorescence detection to increase selectivity. Detection limits obtained from authentic standards range between 1.3 and 4.2 ng/ml and allow sensitive phytoestrogen quantitations (Barnes et al., 1998).

1.3.4 DETECTION

Traditionally, reversed-phase HPLC analysis combined with UV detection has been used to measure isoflavones in soy products because of the high concentration of isoflavones from these sources (Barnes, Kirk, and Coward, 1994; Coward et al.,

TABLE 1.9
Retention Times of Isoflavonoids from Soybean
Byproduct Separated by HPLC

Isoflavones	Retention Time (min)	R _f
Daidzin	21.0	0.41
Glycitin	22.3	0.45
Genistin	25.2	0.43
Glycitein 6- <i>O</i> -acetylglucoside	26.8	0.61
Glycitein 6- <i>O</i> -malonylglucoside	27.4	0.12
Daidzein 6- <i>O</i> -acetylglucoside	27.7	0.56
Genistein acetyl glucoside	29.2	0.59
Daidzein 6- <i>O</i> -malonylglucoside	30.1	0.08
Genistein acetyl glucoside	31.0	nr
Daidzein	32.0	nr
Glycitein 6- <i>O</i> -malonylglucoside	32.5	nr
Genistein 6- <i>O</i> -malonylglucoside	33.0	0.10
Glycitein	34.0	nr
Genistein	37.4	nr

Note: R_f values from Kudou et al., 1991; nr, not reported.

Source: Adapted from Plewa, M. J., et al. *Teratogenesis Carcinog. Mutagen.*, 19, 121–135, 1999.

1993; Eldridge, 1982a; Franke et al., 1995; Wang and Murphy, 1994). However, foods with much lower levels of isoflavones, or those with other phytoestrogens such as coumestrol or the lignans, present a greater technical challenge. The maximum absorption of daidzein is at 249.8 nm with a shoulder at 301.9 nm, while those of genistein and biochanin A are at 259.2 nm (Hutabarat, Mulholland, and Greenfield, 1998). Isoflavones are generally detected at 236 (Graham, 1991a,b), 260 (Garrett et al., 1999), 262 (Barnes, Kirk, and Coward, 1994), and 280 nm (Kinoshita, Ozawa, and Aishima, 1997; Kinoshita et al., 1998). Wang used UV detection at 254 nm and fluorescence detection at 365 and 418 nm for excitation and emission, respectively (Wang et al., 1990). DAD and coulometric detection were used simultaneously for detection of isoflavones in soy foods (Franke et al., 1998).

Fluorescence detection (Franke et al., 1994; Pettersson and Kiessling, 1984; Wang et al., 1990), amperometric detection (Dewald et al., 1991; Kitada et al., 1986; Setchell, Welsh, and Lim, 1987), and thermospray MS with SIM (Dewald et al., 1991; Kitada et al., 1986; Setchell, Welsh, and Lim, 1987) have been very useful in increasing the sensitivity and selectivity of commonly used UV detection (Dziedzic and Dick, 1982; Eldridge, 1982a; Jones, Price, and Fenwick, 1989; Mellenthin and Galensa, 1997; Pettersson and Kiessling, 1984; Van de Casteele, Geiger, and Van Sumere, 1982). Müllner and Sontag (1999) describe a reliable and sensitive method for quantification of soybean phytoestrogens, daidzein and genistein, by HPLC with coulometric electrode array detection using bisphenol A as the internal standard.

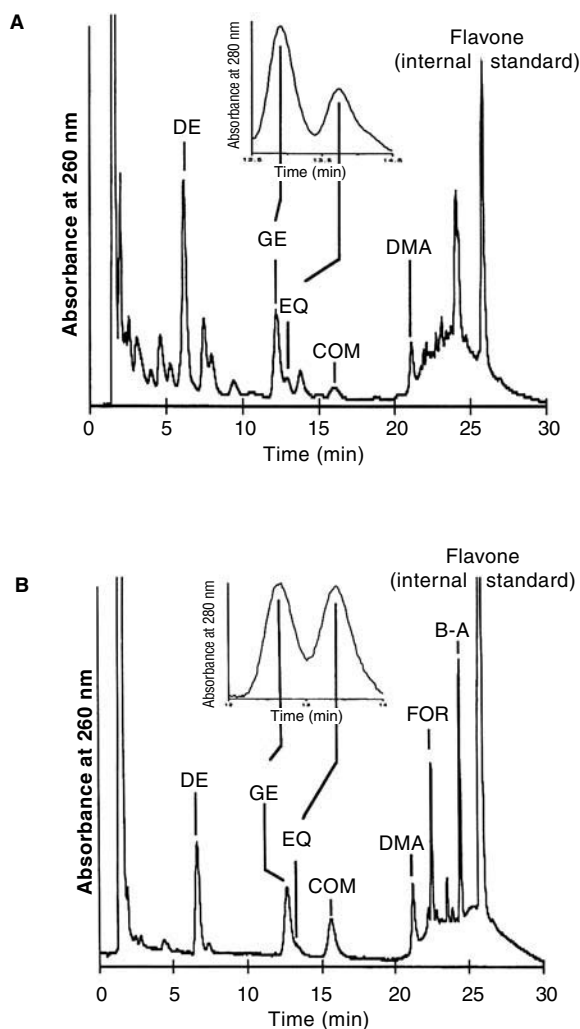


FIGURE 1.8 HPLC separation of urinary phytoestrogens (A) and standards (B) monitored at 260 and at 280 nm (insert). Peak identification: DE, daidzein; GE, genistein; COM, coumestrol; FOR, formononetin; B-A, biochanin A; flavone, internal standard. (Reprinted with permission from Franke, A. A., et al., *Proc. Soc. Exp. Biol. Med.*, 208, 18–26, 1995.)

Samples are acid hydrolyzed (10 M HCl with ethanol) into aglycones and purified in a C₁₈ cartridge, and the phytoestrogen is eluted with methanol (80%). The phytoestrogens are separated on a reversed-phase column (Hypersil® Elite C₁₈ and eluted with methanol–acetonitrile–50 mm sodium acetate pH 4.8 (40:20:40, v/v/v) and detected in a coulometric electrode array detector using an oxidative detection mode (+390 to +810 mV in increments of 60 mV, vs. palladium reference electrode. Daidzein, genistein, and biochanin A have similar recoveries at 95 to 98% and detection limits of 0.9, 1.0, and 1.4 mg/kg, respectively, using this method.

1.3.4.1 Electrochemical Detection

The phytoestrogens can be detected by UV absorption at 260 to 280 nm with a detection limit of about 5 ng injected (signal-to-noise ratio of 3 at 0.002 a.u.f.s.). The phytoestrogens are also electroactive, due to the presence of phenolic groups, and can therefore be detected with electrochemical detection (ED). Coumestrol is the most electroactive compound, followed by genistein and daidzein. The optimum potential for the simultaneous sensitive detection of all three compounds is +0.75 V, and at a sensitivity of 3 nA, the detection limits of coumestrol, genistein, and daidzein are 5, 10, and 15 pg injected, respectively. ED is more sensitive than the UV detector for coumestrol, genistein, and daidzein. Formononetin and biochanin A require a high operating potential ($\geq \pm 1.2$ V) resulting in baseline instability in ED, for the detection of a wide range of phytoestrogens. However, in preparations containing only daidzein and genistein, ED is the obvious detection system of choice since it allows for a much smaller sample size to be used, resulting in a simpler and cleaner matrix. For the specific detection of coumestrol a lower operating potential ($> \pm 0.45$ to 0.5 V) may be used since few compounds are electroactive at these low potentials (Setchell, Welsh, and Lim, 1987).

1.3.4.2 Coulometric Array Detection

A less expensive technique is the combination of HPLC with a coulometric array detector (Gamache, Ryan, and Acworth, 1993). The advantages of this technique are the simplicity of extraction, only hydrolysis or simple alcohol extract is needed, the high selectivity of the detector excluding most contaminant, and the ability to determine conjugated phytoestrogens. Comparisons with GC-MS and immunoassay results using both biological fluids and food samples have shown that this technique in most instances yields specific results and can separate 13 different phytoestrogens and their metabolites in one HPLC run (Adlercreutz, 1999).

Nurmi and Adlercreutz (1999) developed an HPLC method for profiling 13 phytoestrogens and their metabolites particularly for nonsupplemented plasma samples using coulometric electrode array detection. The method is less sensitive compared to GC-MS, but higher than other HPLC methods using diode array or UV detection. Detection limits range from 13.4 (SDG) to 40.3 (genistein) pg on a column. The intra- and interassay range from retention time variations are negligible, and frequent calibration eliminates detector response variation. The accuracy of the method for six analytes ranges from 69% for enterodiol to 118% for genistein. The intra- and interassay precision CV ranges from 1.5 to 14% and 9.9 to 44%, respectively.

Separation of the phytoestrogens is carried out by using gradient elution with sodium acetate buffer (50 mM, pH 5)–MeOH (80:20, v/v) and sodium acetate buffer (50 mM, pH 5)–MeOH–ACN (40:40:20, v/v/v) as eluents A and B, respectively. A low flow rate of 0.3 ml/min results in a long analysis time of 85 min. A pretreatment with β -glucuronidase and sulfatase is required to hydrolyze the conjugated compounds of plasma by incubation at 37°C for 16 h. The sensitivity of coulometric electrode array detection enables the analysis of low-level plasma phytoestrogens. Phytoestrogen metabolism partly explains the difficulties in determining the precision for all compounds in the same sample. This method is useful for monitoring plasma

phytoestrogen profiles in nonsupplemented samples. The higher the concentration of the compound, the better the method precision, even if concentrations close to detection limit determination are satisfactory, when compared to other methods with similar sensitivity.

Gamache and Acworth (1998) applied coulometric array detection which uses a series of flow-through electrochemical sensors, each providing 100% electrolytic efficiency for measurement of phytoestrogens in complex matrices. The binary gradient reversed-phase C₁₈ chromatography consisted of 50 mM sodium acetate buffer (pH 4.8) acetic acid–methanol–acetonitrile solvent system (80:20:0 and 40:40:20, v/v/v). Eight coulometric sensors are set at 260, 320, 380, 440, 500, 560, 620, and 680 mV (vs. Palladium reference electrode). Compounds are resolved in 30 min via both their oxidation/reduction characteristics and their chromatographic behavior. Maximal oxidation potentials (mV) are 380 for coumestrol; 500 for daidzein and genistein; 560 for estradiol, equol, and estriol; 440 for diethylstilbestrol; and 620 for enterodiol, enterolactone, and daidzin with 5 to 50 pg limits for detection. Ethanol extracts of urine samples from human subjects are evaporated and reconstituted in 20% methanol prior to HPLC analysis. Daidzein, enterodiol, enterolactone, equol, and genistein are determined in urine with less than 5% (RSD) interassay imprecision and 85 to 102% recovery.

This technique is based on the use of multiple electrochemical detectors placed in series after the analytical column and maintained at different potentials. Analytes are detectable at 10 pg per injection, and the response is linear over three orders of magnitude for all analytes. Coulometric array detection provides low picogram sensitivity and a wide linear response range, suitable for the measurement of phytoestrogens, with a high degree of selectivity. This technique is easy to use with both isocratic and gradient elution chromatography and may provide an alternative to chromatographic methods that use MS as a means of detection.

1.3.5 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

1.3.5.1 Isoflavones

Liquid chromatography-mass spectrometry (LC-MS) is a powerful technique for analysis of complex mixtures, where additional analytical information is required to positively confirm the identity of the separated compounds or few separations are obtained. Among the interfacing systems used to couple LC with MS, the newly developed electrospray/ion spray mass spectrometric liquid interface allows undoubted advantages in terms of sensitivity and capability to analyze large, thermally labile, and highly polar compounds; in addition, tandem MS techniques are useful for structural elucidation studies.

The use of the hyphenated technique LC-MS provides important advantages because of the combination of the separation capabilities of LC and the power of MS as an identification and conformation method. The combination of LC with various types of MS systems (Barnes et al., 1998; Coward et al., 1996) culminates in advantages of these techniques that need less purification and no derivatization compared to the GC-MS determinations and are relatively specific, particularly the MS-MS techniques. Over LC-MS combinations may not be as specific as the GC-MS method

because of steroids interference when biological samples are analyzed after short purification. The inability of GC-MS to analyze nonvolatile, high polar, and/or thermally unstable substances has led to a growing interest in the development of LC-MS as a valuable analytical technique. In this context LC-MS is a viable system for analyzing naturally occurring compounds such as phytoestrogens in foods and biological fluids.

There are different approaches to the hyphenation of HPLC with MS, and some commercial interfaces are available. Atmospheric pressure ionization (API), a “soft” and highly efficient method suitable for analysis of polar, ionic, high molecular mass, and thermally labile compounds amenable to LC, has greatly increased the popularity of LC-MS. API-based interfacing systems are electrospray (ES) and ionspray (ISP), which are liquid-based interfaces.

Analytical methods utilizing thermospray (TSP) LC-MS have been useful in analyzing phytoestrogens in various soya protein preparations (Setchell, Welsh, and Lim, 1987). Best separations of the phytoestrogens daidzein, genistein, coumestrol, formononetin, and biochanin A are obtained in a reversed-phase mode with methanol–ammonium acetate buffer (0.1 M, pH 4.6; 3:2 v/v) containing 0.25 mM ethylenediaminetetraacetic acid (EDTA) as the mobile phase. Extracts containing isoflavones, their polar conjugates, and related substances obtained after aqueous 80% ethanol extraction of soybean products are submitted to enzymatic hydrolysis before LC analysis. The compounds are positively identified in the chromatography effluent by performing MS analysis in the m/z 100 to 300 range; daidzein and genistein are detected in high concentrations in soybean milk formulas, soybean flakes, and textured soybean protein. Optimum ionization of phytoestrogens can be achieved by operating the mass spectrometer in a continuous scanning mode over a mass range of 100 to 300 Da with vaporizer and jet-block temperatures of 135° and 215°C, respectively (Setchell, Welsh, and Lim, 1987). In another study of the same group (Barbuch et al., 1989) TSP-MS-MS proved to be a useful technique, yielding valuable structural information absent from the mass spectra of the phytoestrogens recorded under TSP-MS conditions. The resultant product ion spectra contained fragments characteristics of each phytoestrogen subclass, allowing the use of tandem MS to confirm identification and propose structures for unknown compounds. TSP-MS-MS analysis of these substances has also been carried out in the neutral loss operating mode by monitoring the neutral loss of ion (due to consecutive releases of CO), common to all members of this family. Several soy protein preparations have been investigated to confirm the presence or absence of phytoestrogens; in one soybean product analyzed, daidzein, genistein, and an unknown phytoestrogen of the biochanin A subclass were found; this unknown phytoestrogen was tentatively identified as 6,7-dihydroxy-4'-methoxyisoflavone using its product spectrum.

HPLC-TSP-MS has been investigated for its potential to identify individual phytoestrogens in the HPLC effluent. When TSP ionization is interfaced with HPLC-MS, ions are formed by spraying the column eluate at a high enough heat to evaporate the solvent. The mass spectra generated in the TSP ionization process are characterized (Figure 1.9) by intense protonated molecular ions $[MH^+]$ with no significant fragmentation of the molecule. Since most of the ionization resides in a single ion, SIM of the $[MH^+]$ for each phytoestrogen affords a more specific method of detecting

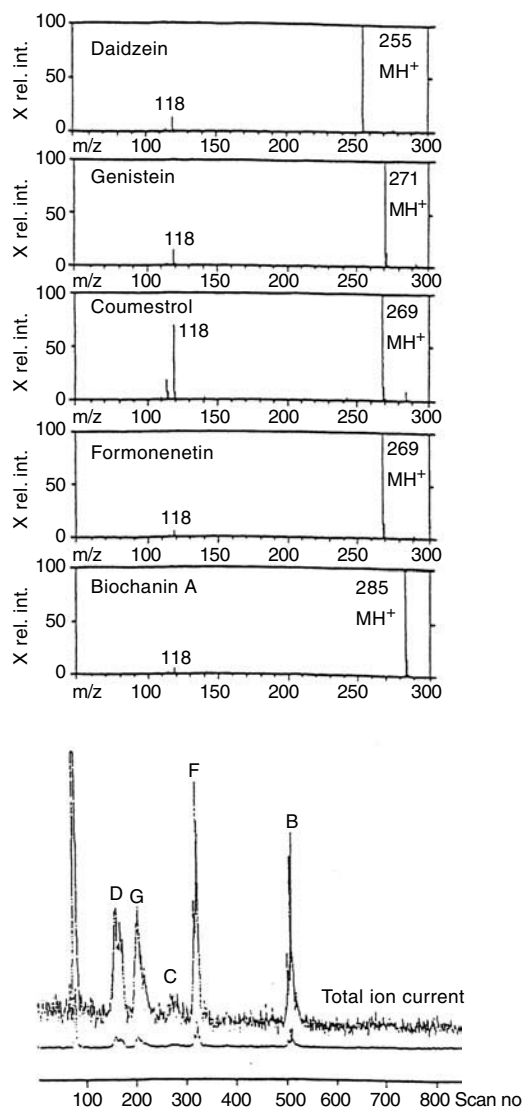


FIGURE 1.9 Mass spectra and total ion current chromatogram obtained for the HPLC-TSP-MS analysis of a mixture of phytoestrogen standards daidzein (D), genistein (G), coumestrol (C), formononetin (F), and biochanin A (B). (Reprinted with permission from Setchell, K. D. R., Welsh, M. B., and Lim, C. K., *J. Chromatogr.*, 386, 315–323, 1987.)

these compounds with a 100-fold improvement in sensitivity over the scanning mode or UV detection alone. It is also suggested that these compounds would be ideally suited to HPLC-MS-MS detection, where after focusing the [MH⁺] ion, collision-induced dissociation would yield fragmentation specific for each compound, thereby assisting structural elucidation of these and unknown phytoestrogens or metabolites separated by HPLC (Setchell, Welsh, and Lim, 1987).

Recently, HPLC with atmospheric pressure chemical ionization (APCI) MS and ISP-MS has been investigated for the first time for the analysis of isoflavones and their conjugates in soy food products (Barnes, Kirk, and Coward, 1994). The analytes are detected in both positive ion (PI) and negative ion (NI) mode using the heated nebulizer APCI and the ISP interfaces. The more valuable spectral data about each isoflavone conjugate are obtained using the heated nebulizer-atmospheric pressure chemical ionization (HN-APCI) system in the positive ion mode. Enhanced sensitivity has also been observed for the positive isoflavone aglucone ions produced in the HN-APCI interface; these ions are about 1.5 to 3 times more intense than the protonated molecules generated in the ISP interface. The effect of mobile phases containing 0.1% acetic acid or 10 mM ammonium acetate is also described as for the major ions detected in the HN-APCI negative ion mass spectra of the isoflavone conjugates (Table 1.10).

LC-MS of two isoflavonoids in a licorice root extract powder using a particle beam (PB) interface has been described (Weinberg et al., 1992). The LC-PB-MS approach identified formononetin and isoliquiritigenin in the LC eluates on the basis of the corresponding PB-EI mass spectra. In the case of the GC-MS method, the corresponding mono- or disilated derivatives are required since the involatility of the analytes are not amenable to direct analysis by GC-MS (Figure 1.10). HPLC has also been coupled to photodiode array (PDA) and to MS using APCI or electrospray ionization (ESI) (HPLC-APCI-CAD-MS or ESI-CAD-MS) for identification of glycosides and HPLC-APCI-CAD-MS for identification of aglycones in investigating the isoflavones of the roots of *Pueraria lobata* (Willd) Ohwi (*Pueraria radix*) (Rong et al., 1998).

TSP as an HPLC-MS interface has been largely superseded by API because of its recognized limitations in robustness and stability of the ion beam. The API techniques of APCI and electrospray ionization (ESI) are highly sensitive, show greater ionization stability, and are more universally applicable than other HPLC-MS techniques. Both APCI and ESI involve the formation and ionization of an aerosol at atmospheric pressure. The suitability of the APCI technique, where spray formed by pneumatic nebulization from a capillary in a heated probe is ionized by a high-voltage corona discharge, has been demonstrated in the determination of isoflavones in plasma (Coward et al., 1996). Aramendia et al. (1995a) have examined synthetic mixtures of isoflavones by capillary electrophoresis ESI-MS using the negative ionization mode.

Using reversed-phase HPLC interfaced with ESI to a mass spectrometer, it is possible to obtain a mass/intensity map of all isoflavone metabolites in a single 20-min analysis. Analysis of isoflavonoid conjugate in serum/plasma samples requires initial extraction, but the conjugates can be measured intact either by capillary reversed-phase HPLC-ESI-MS or on regular reversed-phase columns by HPLC-HN-APCI-MS. When it is only necessary to measure the total isoflavonoids and their metabolites in blood, hydrolysis can be performed directly in serum/plasma samples, and flavonoids can be recovered by ether acetate solvent extraction (Barnes et al., 1998).

In the HN-APCI interface, the mobile phase is passed down a quartz tube heated to 400° to 500°C. Ionization is accomplished by first ionizing the air within the

TABLE 1.10
Ions Observed for Isoflavone Conjugates by Atmospheric Pressure Chemical Ionization-Mass Spectrometry in the Negative Mode^a

Isoflavone ^b	<i>m/z</i>	Relative Abundance	
		10 mM NH ₄ OAc	0.1% Acetic Acid
Daidzein 6-OMalGlc	517 (M-COOH + CH, COOH) ⁻	36.5	66.7
	457 (M-COOH) ⁻	14.2	32.6
	253 (M-H-MalGlc) ⁻	100.0	100.0
Daidzein 6-OAcGlc	517 (M-H + CH, COOH) ⁻	40.7	63.8
	457 (M-H) ⁻	16.4	30.8
	253 (M-H-AcGlc) ⁻	100.0	100.0
Daidzein	475 (M-H + CH, COOH) ⁻	24.1	100.0
	451 (M-H) ⁻	8.5	39.5
	253 (M-Glc) ⁻	100.0	100.0
Genistein 6-OMalGlc	533 (M-COOH + CH, COOH) ⁻	40.1	67.8
	473 (M-COOH) ⁻	46.0	70.3
	269 (M-H-MalGlc) ⁻	100.0	100.0
Genistein 6-OAcGlc	533 (M-H + CH, COOH) ⁻	9.1	85.5
	473 (M-H) ⁻	25.2	100.0
	269 (M-H-AcGlc) ⁻	100.0	27.5
Genistein	491 (M-H + CH, COOH) ⁻	25.5	69.2
	431 (M-H) ⁻	28.1	100.0
	269 (M-H-Glc) ⁻	100.0	94.2
Glycitein 6-OMalGlc	547 (M-COOH + CH, COOH) ⁻	2.0	13.5
	487 (M-COOH) ⁻	9.3	29.2
	283 (M-H-MalGlc) ⁻	100.0	100.0
Glycitein 6-OAcGlc	547 (M-H + CH, COOH) ⁻	17.5	100.0
	487 (M-H) ⁻	7.7	49.3
	283 (M-H-AcGlc) ⁻	100.0	34.8
Glycitin	505 (M-H + CH, COOH) ⁻	5.1	82.6
	445 (M-H) ⁻	1.8	29.7
	283 (M-H-Glc) ⁻	100.0	100.0

^a Mass spectra obtained during HPLC analysis (in a background of 10 mM ammonium acetate or 0.1% acetic acid) of an 80% aqueous methanol extract of toasted soy flour using the HN-APCI interface. Orifice potential was -60 V.

^b 6-OMalGlc, 6''-O-malonylglucoside; 6-OAcGlc, 6''-O-acetylglucoside.

Source: From Barnes, S., Kirk, M., and Coward, L., *J. Agric. Food Chem.*, 42, 2466-2474, 1994. With permission.

interface by a corona discharge needle. In this type of interface, sensitivity is determined by the total mass of solutes present, rather than their concentration as in the case of ESI-MS. Solutes obtained from the fractionation of isoflavonoids and other phytoestrogens generally extracted with 80% aqueous and separated on an Aquapore C₈ reversed-phase HPLC column can be introduced directly into the mass spectrometer via the HN-APCI interface operating in both the positive and the

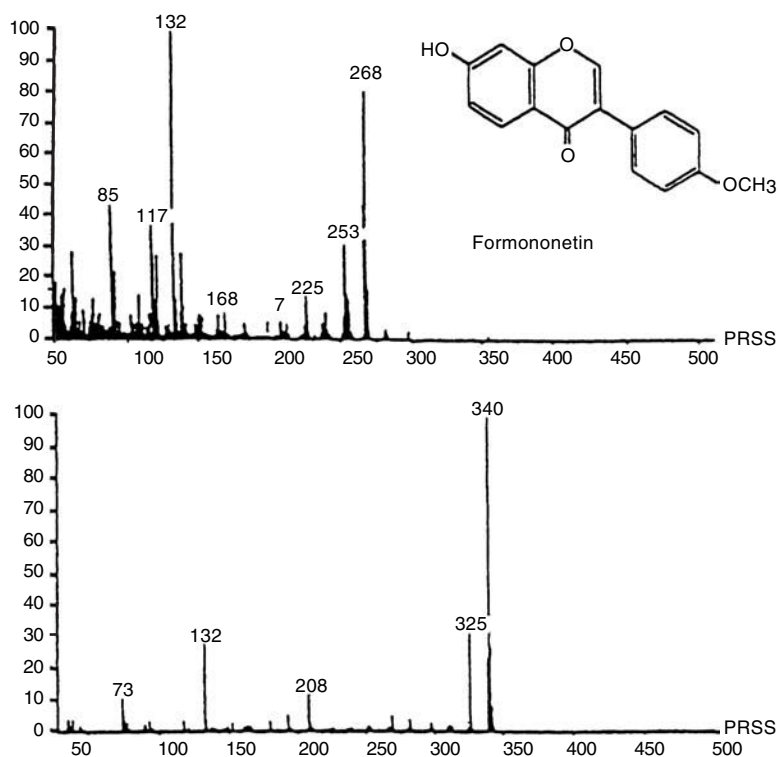


FIGURE 1.10 Mass spectra of formononetin obtained by LC-MS (top) and monosilylated formononetin obtained by GC-MS (bottom). (Reprinted with permission from Weinberg et al., 1992.)

negative modes. In the scan mode, ions entering the mass spectrometer are analyzed over an m/z range from 50 to 800. MS-MS daughter ion spectra are obtained by passing molecular ions selected by the first quadrupole into an argon gas collision cell and analyzing the fragment ions in a third quadrupole (Barnes et al., 1998).

Measurement of the isoflavonoids and their metabolites in urine from those consuming a heavy soy diet can be carried out by HPLC-UV analysis (Franke and Custer, 1994) as well as HPLC-MS. In the latter method, multiple reaction ion monitoring (MRM) is used, combining each parent molecular ion with a specific daughter ion produced by collision-induced dissociation. The data from the two methods are significantly correlated ($r > 0.92$, $p \leq 0.0001$). However, the concentrations of daidzein detected by the HPLC-HN-APCI-MRM method are 40% lower than those by HPLC-UV. Thus, at low urinary isoflavone concentration found in those consuming a regular American diet, HPLC-UV is inadequate, and HPLC-MS becomes the method of choice. In plasma and serum, where isoflavone concentrations are low ($<1 \mu M$), a high-pressure collision cell is used for extensive fragmentation of the ions with a tenfold increase in sensitivity. Because of the high selectivity of the parent-daughter ion combination, the chromatographic separation time is reduced from 25 to 30 min to 6 min and reproducibility is improved under isocratic conditions.

HPLC-MS using ESI and HN-APCI interfaces is highly applicable to the analysis of isoflavones and their metabolites in foods and biological fluids. The interfaces permit the detection of diagnostic ions without the need for derivatization and multistep extraction procedures. Analysis of isoflavones has also been carried out using the HN-APCI interface (Aramendia et al., 1994; Barnes, Kirk, and Coward, 1994; Coward et al., 1996). For this procedure the instrument is initially calibrated in the positive APCI mode using a mixture of polyethylene glycols over the mass range m/z 80 to 1100. Tuning is then optimized in ESI on the m/z 42 background ion followed by optimization on the protonated molecule $[M + H]^+$ ions of daidzein and genistein at m/z 255 and 271. Typical operating conditions are capillary voltage 2.4 kV, cone voltage offset 5 V, high-voltage lens 0 kV, and source temperature 150°C. Scanned acquisitions are made over the range m/z 80 to 620 with a scan time of 2 sec (Figure 1.11). At low voltage (20 V), daidzin and genistin loss of the glucose molecule occurs readily to yield the corresponding aglycone masses at m/z 255 and 271, respectively. Detection is based on a single ion and the retention time because fragmentation is precluded due to the highly conjugated nature of the molecule. This interface is well suited to simply applying previous HPLC methods in which isoflavonoids are detected by their UV absorbance. Identification of physiological conjugates (β -glucuronides, sulfates) using the HN-APCI interface can only be based on chromatographic mobility.

Barnes et al. (1998) developed a microbore HPLC-ESI-MS method for the determination of the phytoestrogens daidzein and genistein in soya flours and baby foods. In this procedure, the samples are hydrolyzed and extracted with acetonitrile–water prior to analysis. Liquid chromatography is performed in a microbore Primesphere 5C₈ using a water–acetonitrile–acetic acid mobile phase at a flow rate of 60 μ l/min and detection is by API in the form of pneumatically assisted ESI-MS. The limit of detection for daidzein and genistein in the flour and food samples is 0.2 and 0.7 mg/kg, respectively. The method is very robust and reliable when operated over a long time period, generating precision data with 4 to 15% coefficient of variation. HPLC coupled with MS such as ESI of treated nebulizer APCI can directly access the intact molecular weight of isoflavones, both conjugated and unconjugated (Aramendia et al., 1995a; Barnes, Kirk, and Coward, 1994; Barnes et al., 1998).

Although HPLC is currently the most widely used analytical technique to quantify isoflavones (Murphy et al., 1997; Song et al., 1998), it is time consuming with tedious sample preparation. In order to overcome these problems, Wang and Sporns (2000) developed a simple protocol for the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) to analyze isoflavones from food samples with minimal sample preparation. MALDI-TOF-MS advantages over other methodologies include speed of analysis (spectrum is obtained in minutes), high sensitivity, wide applicability combined with good tolerance toward contaminants, and the ability to analyze complex mixtures (Karas, 1996).

For the MALDI-TOF-MS procedure, acetonitrile crude extract of isoflavone is diluted tenfold and loaded on Sep-Pak C₁₈ cartridges where the isoflavones are retained and later eluted with 70% methanol. The eluent is applied directly to MALDI-TOF-MS to acquire the isoflavone spectrum of interest. In MALDI-TOF-MS, isoflavones exhibit only fragmentation corresponding to loss of their carbohydrate residues.

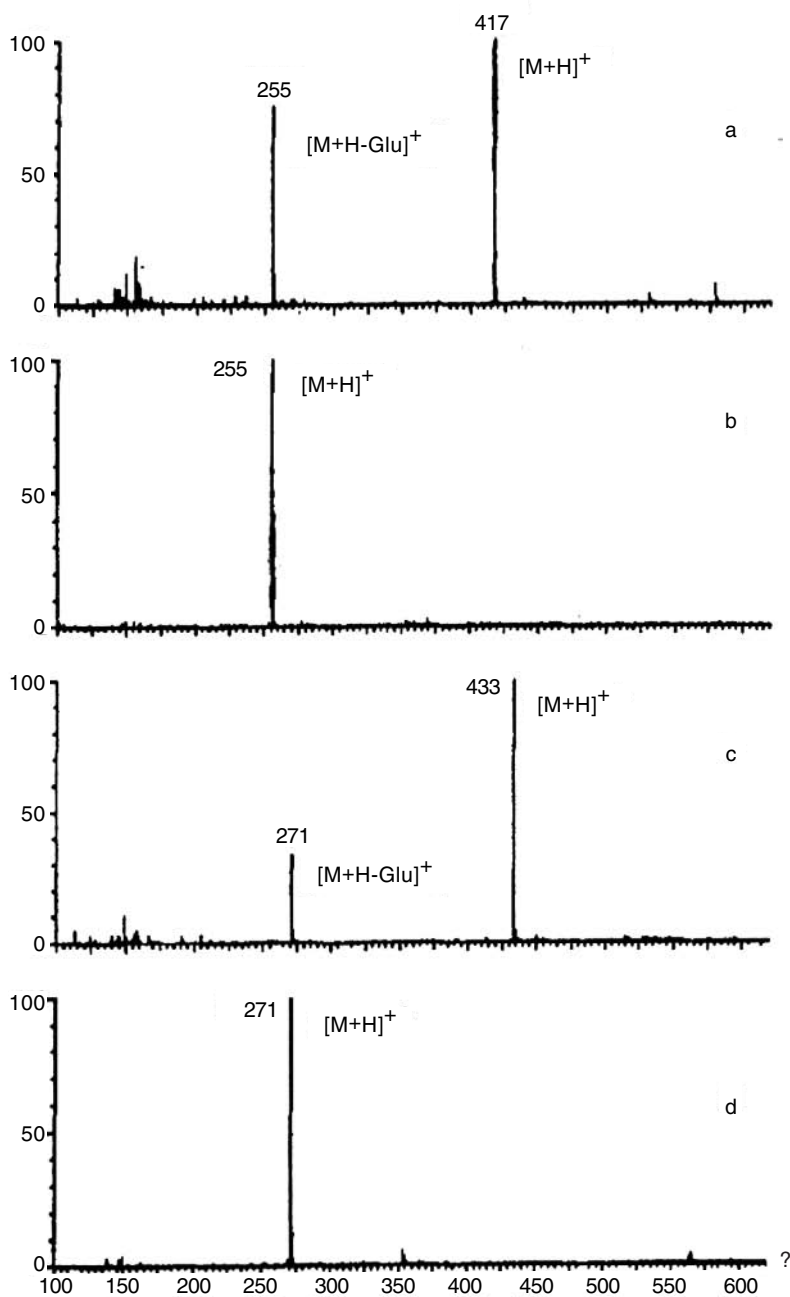


FIGURE 1.11 HPLC-ESI-MS of (a) daidzin, (b) daidzein, (c) genistin, and (d) genistein obtained at low cone voltages. (Reprinted with permission from Barnes, S. et al., *Proc. Soc. Exp. Biol. Med.*, 217, 254–262, 1998.)

Daidzin and genistin fragment and produce $[M-162 + H]^+$ ions at m/z 255 and 271, respectively, similar to the fragmentation pattern observed in ESI-MS (Barnes et al., 1998). The response of daidzin in MALDI-TOF-MS is more than twice that of genistin. MALDI-TOF-MS identification of isoflavones is similar in retention time and peak positions to those obtained by HPLC methods (Wang and Sporns, 2000). MALDI-TOF-MS can easily study changes in isoflavones due to processing or can serve as a rapid analytical tool for authenticity.

1.3.5.2 Lignans

At least six different methods for analysis of lignans in flaxseed and products containing flaxseed, and three for analysis of lignan metabolites in mammalian tissues and fluids have been proposed since the first lignan SDG was first isolated from flax by Bakke and Klosterman (1956). In addition, several variations that exist for many of these methods in the extraction of lignans from flaxseed and products containing flaxseed further complicate its analysis. Results from these studies have been inconsistent, mainly due to differences in methodology.

Lignans in flaxseed, similar to isoflavones in soybeans, mostly occur in the conjugated forms as an ester-linked complex; hence, extraction conditions require conversion to aglycones prior to analysis. Several of the commonly used extraction procedures are inefficient in extracting lignans from plant materials (Table 1.11). Most extraction methods involve the use of a combination of alcohol and another solvent. The β -glucuronidase enzyme is also used to extract lignans as a first step in a multistep hydrolysis process. A comparison of enzymatic hydrolysis of lignans vs. extraction and chemical hydrolysis of lignans has shown that more lignans can be detected with extraction and chemical hydrolysis than with enzymatic release (Westcott, Muir, and Northrup, 1998).

In mammalian tissues lignans have been commonly analyzed by GC (Fotsis et al., 1982) and later by GC-MS (Adlercreutz et al., 1995), although these compounds can be more appropriately assessed by HPLC. A significant limitation of lignan analysis by GC-MS resides in the insufficient volatility of the parent compound without derivatization and the conversion of the conjugates to their corresponding aglycones. These limitations have been overcome by devising a series of hydrolysis and purification procedures (Adlercreutz et al., 1993). However, quantitation becomes increasingly difficult, especially when complicated purification procedures are employed in GC-MS methods, unless internal standards are used. The preferred method is the use of $2H$ -labeled aglycones and SIM to increase sensitivity (Adlercreutz et al., 1993). However, only compounds for which internal standards are available, such as enterodiols, enterolactone, and deuterated matairesinol, have been reported in many studies.

Analytical systems as an alternative to the costly mass spectrometers or improvement to the sensitivity of HPLC systems has been proposed. GC-ion mobility, an atmospheric pressure detection system for GC, has been used to detect the presence of mammalian lignans in urine samples (Atkinson, Hill, and Shultz, 1993). Recently, ED has been used to determine enterolactone and enterodiol in picomole concentrations

TABLE 1.11
Extraction Systems for Lignan Isolation from Flaxseed
and Flaxseed-Containing Foods

Extraction	Hydrolysis	Purification	Level SDG ($\mu\text{mol/g}$ seed)	Ref.
MeOH–dioxane (1:1) 24 h	Ba methoxide	Cellulose column	3.15	Bakke and Klosterman, 1956
<i>In vitro</i> fermentation	Na methoxide	Silical gel (CHCl_3 –MeOH– H_2O)	0.96–3.15	Thompson et al., 1991
β -Glucuronidase	β -Glucuronidase	C_{18} SPE	1.19–1.97	Obermeyer et al., 1995
β -Glucuronidase	β -Glucuronidase + 2 M HCl, 2.5 h, 100°C	Ether extraction/DEAE- Sephadex OH^- QAE-Sephadex Ac^-	9.05–10.21	Mazur and Adlercreutz, 1998
Reflux, 80% MeOH, 2 h	β -Glucuronidase	C_{18} SPE + lipophilic gel chromatography	0.22–3.41	Setchell et al., 1999
70% Aqueous alcohol	NaOH	C_{18} SPE	5.24–15.74	Westcott and Muir, 1998
95% EtOH–dioxane (1:1) 8 h	nr	nr	0.001–0.004	Harris et al., 1994
SCO_2 + THF– H_2O (1:1)	nr	nr	7.15	Wilson et al., 1993
Shaker, 80% MeOH, 4 h, 55°C	1 M HCl, 1 h, 100°C	EtOAc–hexane (1:1)	nr	Meagher et al., 1999

Note: nr, not reported.

Source: Adapted from Muir et al. (2000).

only after the conversion of the conjugates to their corresponding aglycones (Gamache, Ryan, and Acworth, 1999).

A new standard method for analysis of lignans in flaxseed and products containing flaxseed has been proposed (Muir et al., 2000) and is outlined in Figure 1.12. Extraction is carried out with aqueous alcohol, either ethanol or methanol, with a liquid to solid ratio of 20:1. The isolated ester complex is hydrolyzed with a dilute base for 3 h at room temperature, neutralized (0.5 ml 0.5 N HAC), filtered, concentrated if necessary, and subjected to HPLC analysis. Quantitation is achieved by using SDG as the external standard. When higher sensitivity is required, samples can be analyzed by LC-MS using soft ionization techniques such as ESI or APCI. Results are expressed as milligrams of SDG per gram of sample or preferably in micromoles per gram of sample.

LC-MS techniques with soft ionization such as ESI and APCI overcome the time consuming and large sample requirements of GC-MS for analysis of lignans in urine and plasma. The urine sample (in MeOH, 1:1, v/v) can be analyzed directly

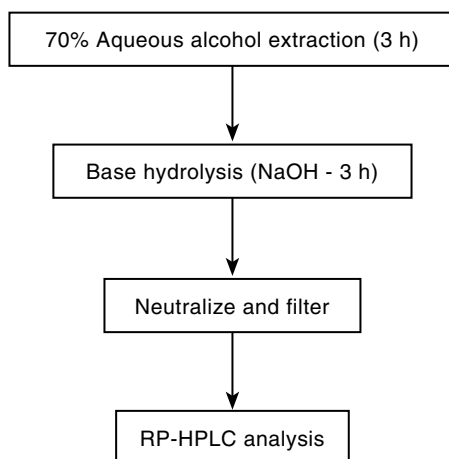


FIGURE 1.12 Flow chart of extraction of lignans from flax-containing samples. (Reprinted with permission from Muir, A. D., et al., Proceedings of the 58th Flax Institute of the United States, Fargo, ND, 2000, pp. 23–32.)

by ESI-MS in the negative ion mode without chromatographic separation (Figure 1.13). Four principle ions, m/z 477, 473, 381, and 377, are detected and further fragmented by collision with argon-producing daughter ions consistent with derivatives of enterodiol (mw = 302 from m/z 477 and 381 fragments) and enterolactone (mw = 297 from m/z 473 and 377), respectively. The mass loss suggests that m/z 477 and 473 are the glucuronides and m/z 381 and 377 are the monosulfates of enterodiol and enterolactone, respectively.

While ESI is probably the most widely used soft ionization technique, APCI can also be used to quantitate lignan metabolites. The sample is chromatographed on a C_{18} column and the effluent is subjected to APCI in the negative ion mode, producing a fragmentation pattern similar to that obtained by ESI. This mammalian lignan conjugates in biological fluids can be identified simply using LC-MS (ESI or APCI) either by direct injection of the sample or after chromatographic separation prior to mass spectroscopic analysis.

The isolation and characterization of potent phytoestrogens, the lignans isolariciresinol, pinoresinol, SDG, and matairesinol, from flaxseed meal has been described by Meagher et al. (1999). The extraction method combined the removal of the lignan glycosides from the plant matrix with an alcoholic solvent system (methanol–water, 80:20 for 4 h at 55°C), followed by acid (1 *M* HCl) hydrolyses (1 h at 100°C) to release the aglycones. The acid-hydrolyzed methanolic extract is separated by reversed-phase HPLC, and lignans are detected at 280 nm with diode array detection. GC-MS is used to characterize the lignans after derivatization. Lignan aglycones isolated from flaxseed have shorter retention times on GC than on HPLC (Table 1.12) (Meagher et al., 1999).

Acid hydrolysis of methanolic lignan extract leads to the isolation of divanillyltetrahydrofuran (anhydrosecoisolariciresinol), previously reported (Mazur et al., 1996) as an artifact of acid hydrolysis resulting from the dehydration of SDG, but

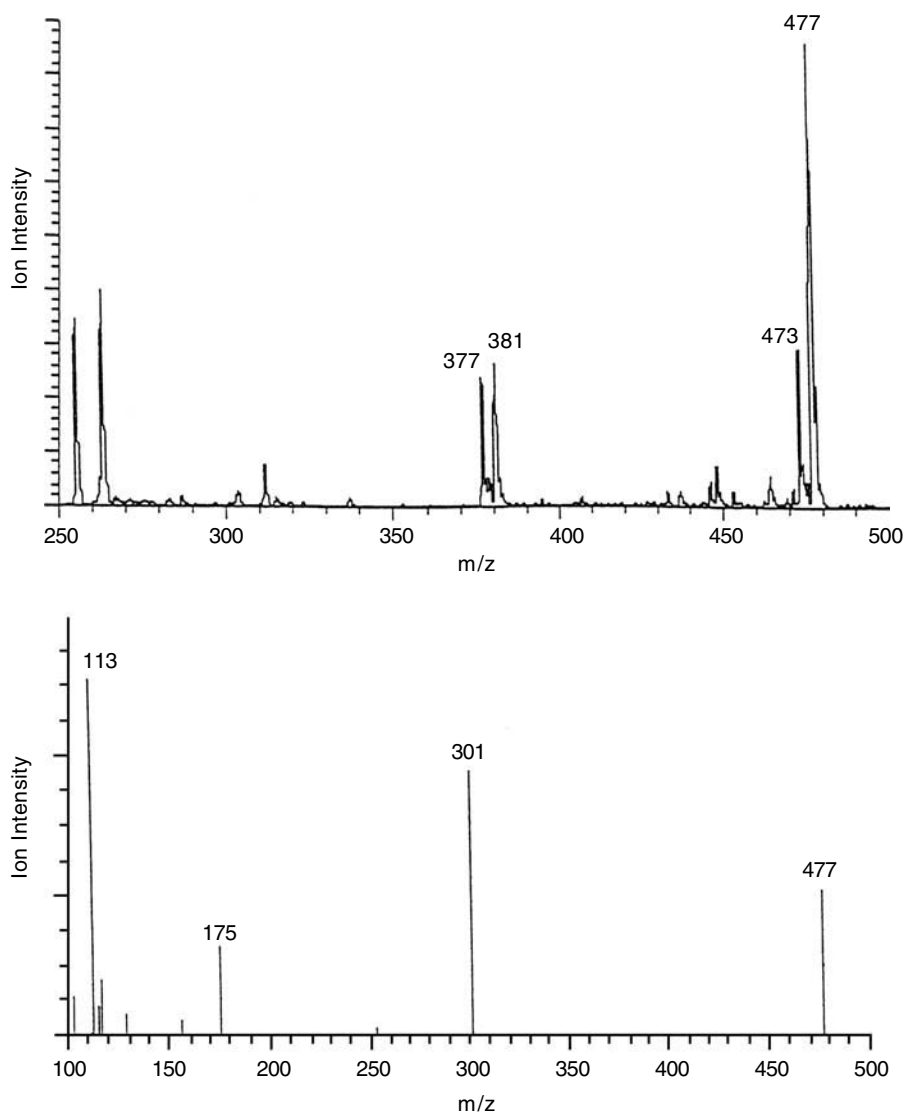


FIGURE 1.13 Direct ESI-MS of urine sample of rabbit maintained on a diet containing 15% flaxseed. Negative ion ES of rabbit urine (top) for ions corresponding to the glucuronides of enterodiols ($m/z = 477$) and enterolactone ($m/z = 473$) and their corresponding sulfates ($m/z = 381$) and ($m/z = 377$). Daughter ion scan of m/z 477 (bottom). (Reprinted with permission from Muir, A. D., et al., Proceedings of the 58th Flax Institute of the United States, Fargo, ND, 2000, pp. 23–32.)

now proven to be a naturally occurring lignan (Meagher et al., 1999). Enzyme hydrolysis of defatted flaxseed meal is not efficient at removing lignan from the plant matrix compared to extraction with an organic solvent system. Combination of aqueous methanolic extraction and subsequent acid hydrolysis renders a lignan

TABLE 1.12
Comparison of Retention Times (Minutes) for Lignan
Aglycons by HPLC-DAD with Those of the Lignan TMS
Derivatives by GC-MS

	GC-MS		HPLC-UV	
	Isolated	Standard	Isolated	Standard
NDGA ^a		8.01		44.3
Isolariciresinol	8.32		^b	
Secoisolariciresinol	8.44	8.41	16.8	16.9
Anhydroseco ^a	9.04		36.0	36.2
Matairesinol	10.54	10.48	31.6	31.5
Lariciresinol		10.01		18.6
Hinokinin		10.55		17.9
Arctigenin		11.13		40.5
Pinoresinol	11.19	11.17	26.8	25.9

^a NDGA, nordihydroguaiaretic acid; anhydroseco, divanillyltetrahydrofuran.

^b Isolariciresinol was not resolved on HPLC due to a coeluting sterol peak.

Source: From Meagher, L. P. et al., *J. Agric. Food Chem.*, 47, 3173–3180, 1999. With permission.

extract that can be further purified by partitioning with ethyl acetate and hexanes. Purification of the HPLC fractions prior to GC-MS analysis is not required since the crude lignan extract can be directly applied to GC-MS without the need for a C₁₈ preparative column or an ion-exchange column used in other reported methods (Mazur et al., 1996; Setchell et al., 1983) as the lignan peaks of interest are well resolved on the nonpolar column.

The most recent analytical method as described by Johnson et al. (2000) involves extraction of defatted flaxseed flour with dioxane/ethanol, aqueous base hydrolysis, solid-phase purification of an SDG-containing fraction, and quantitative analysis by HPLC. After base hydrolysis and release of SDG from its polymer with aqueous NaOH, the extracts are acidified to pH 3 to prevent ionization of the carboxylic and phenolic groups. The acidification leads to the formation of salts that are removed by SPE in a C₁₈ reversed-SPE column with almost quantitative recovery (>99.5%) of SDG from the SPE column.

The gradient consisting of solvent A (5% acetonitrile in 0.01 M phosphate buffer, pH 2.8) and solvent B (acetonitrile) mixed solvents A and B [(v/v): 0 min (100:0), 30 min (70:30), and 32 min (30:70) at 1 ml/min] provides good separation of SDG and requires less time than other HPLC gradients (Figure 1.14). In this system SDG elutes at 19.5 min with *p*-coumaric acid and ferulic acid glucoside eluting at 12 and 15 min, respectively. The yield of SDG is independent of the solvent (methanol or water) used for base hydrolysis. This study is the first to provide data on the variation in SDG content in whole flaxseeds (6 to 13.3 mg/g). The nature and utility of the SDG polymer in animal or human nutrition has not been reported.

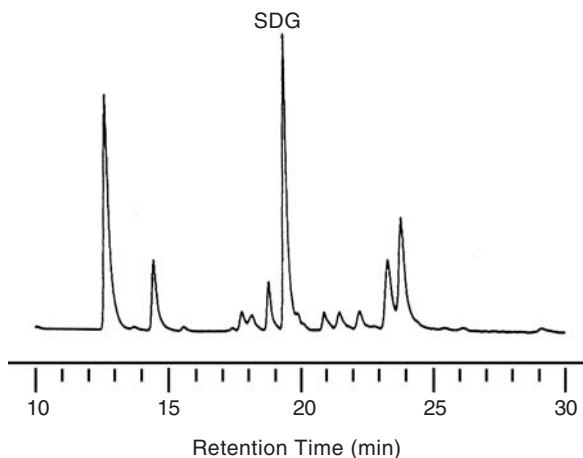


FIGURE 1.14 HPLC of flaxseed hydrolyzate recorded at 280 nm with a diode array detector. (Reprinted with permission from Johnson, P., et al., *J. Agric. Food Chem.*, 48, 5216–5219, 2000.)

1.3.5.3 Ionspray Mass Spectrometry (ISP-MS) of Lignans

ISP-MS and tandem MS (MS-MS) have been used to rapidly detect and unambiguously identify secolarisiresinol in flaxseed (Bambagiotti-Alberti et al., 1994a). According to the authors, SDG is present only in the methanolized extract, suggesting that it is complexed and embedded in flaxseed. Detection of SDG is possible within the product arising from methanolysis, bypassing any chromatographic separation. The only drawback is the lack of structural information, this being typical of soft ionization techniques such as ISP.

In this procedure, air-dried hexane-defatted flaxseed meal is extracted with a methanol–dioxane mixture (1:1) and analyzed by MS flow injection analysis (FIA). The methanol–dioxane extract is methanolized with methanol and sodium methoxide, neutralized with acid (1 *N* H₂SO₄), filtered, and analyzed with FIA-MS and MS-MS. The molecular weight of SDG is 686 Da.

Later, Bambagiotti-Alberti et al. (1994b) set out to test the comparative performance of LC-ISI-MS (ionspray ionization mass spectrometry) and LC-CF-FAB-MS (liquid chromatography continuous-flow fast-atom bombardment mass spectrometry) of the two isomeric forms of the lignan SDG. The liquid chromatographic separation is performed using a gradient of acetonitrile and water containing TFA (0 to 30%). The eluate is introduced into the ISI interface by splitting after the UV detector. The ISI total ion current (TIC, mass range *m/z* 600 to 800) and mass chromatograms of methanolized dioxane-methanol extract of defatted flaxseed meal shows two peaks corresponding to the two isomers of SDG present in flaxseed.

A comparison of performance achieved in the analysis of lignans of ISI and CF-FAB reveals that in the positive ion mode, ISI provides stable quasimolecular ion species which, by MS-MS, give fully diagnostic product ion spectra. ISI is seen to

be almost useless in the negative ion mode since it fails to produce CID-prone (collision-induced decomposition) ion species. CF-FAB, on the other hand, gives rise to spontaneous diagnostic fragmentation along with the expected quasi-molecular species, allowing positive identification without MS-MS intervention. However, in the positive ion mode, a dense spectral background can hamper recognition of the diagnostic peaks, especially at low analytic concentrations. In the negative ion mode, the peaks of interest lie on a smooth background.

1.3.6 CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE) is a powerful technique that affords rapid, high-resolution separations (10^4 to 10^6 theoretical plates) while requiring only a few femtomoles of sample. The technique is applicable to a wide range of analytes present in buffered aqueous solution as charged species. The utility of CE, however, is greatly enhanced by MS detection, particularly with a soft ionization technique such as ESI to produce ions even from thermally labile, nonvolatile, polar compounds.

The mass spectrometer equipped with an ESI source is operated in the negative ion mode at a probe tip voltage of -3.5 kV. The extraction cone voltage serving primarily to focus ions into the mass analyzer varies from -25 to -75 V. As a general rule, compounds with a methoxy group (biochanin A and formononetin) exhibit one fragment ion at m/z $[M-H-CH_3]$ when high extraction voltages are used. The mass spectrometer is scanned from m/z 90 to 325 at 25 s per scan. A coaxial sheath liquid consisting of water-2-propanol (80:20) at a flow rate of 10 μ l/min is the fluid for CE-ESI-MS. Nitrogen is used as both a drying gas (50 l/h) and the ES nebulizing gas (10 l/h). Mass spectral data are acquired using selected-ion recording (SIR mode; 0.2 s dwell time, 0.2 mass unit span) for the $[M-H]$ ion. For CE, samples are electrophoresed at 305 V/cm, resulting in very stable currents of about 35 μ a, and detected by UV at 260 nm with scanning between 190 and 400 nm (Aramendia et al., 1995a).

Analysis with CE is complete within 4 min (Figure 1.15) for an isoflavone mixture (1 mM) containing about 3 fmol of each compound after 20 cm of capillary using UV detection at 260 nm. The signal reproducibility is very good with less than 10% peak variation for the same sample. The success of the CE-ESI-MS analysis of isoflavones relies on many factors, including the ESI interface and buffer composition. Analyte detectability and sensitivity vary widely with buffer concentration; the optimum signal is obtained at the lowest possible concentration (10 to 25 mM). CE with its exceptionally low flow rate (nanoliters per minute) is easier to interface to MS than to LC, since no flow splitting is required.

Dietary phytoestrogens have been determined by a fast and selective CE method. In this procedure, the isoflavones daidzein and genistein are separated on an uncoated fused-silica column using borate buffer and diode array detection at 254 and 268 nm, respectively. The linear response ranges from 5 to 100 mg/dm^3 , with a minimum detectable limit at 0.4 mg/dm^3 for both analytes. The relative response factors for daidzein and genistein are 0.519 and 0.755, respectively, when *p*-nitrophenol is used as an internal standard (Vänttinen and Moravcova, 1999). Aramendia et al. (1995a) separated isoflavones on an uncoated fused-silica CE column (110 cm $\times$ 75 μ m I.D.)

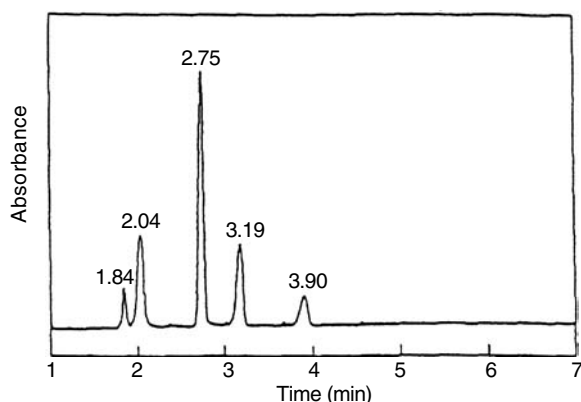


FIGURE 1.15 Electropherogram of isoflavone mixture with UV detection of 260 nm. Peaks: genistein (1.89 min); daidzein (2.04 min); pseudobaptigenin, formononetin, and biochanin A (2.76 min); isoliquirtigenin (3.19 min); and biochanin A 7-glucoside (3.90 min). (Reprinted with permission from Vääntinen, K. and Moravcova, J., *Czech. J. Food Sci.*, 17, 61–67, 1999.)

using ammonium acetate buffer (25 ml) and UV and ESI-MS detection. ESI-MS enables the determination of the molecular mass of isoflavones and also the presence of various functional groups according to observed losses from the $[M-H]$ ion during collision-induced dissociation by adjusting some MS parameters.

For quantitative investigations using CZE, an addition of 3-isobutyl-1-methylxanthin as an internal standard is recommended. The CZE separations are performed on a fused-silica capillary of 50 or 70 μm I.D. with boric acid adjusted to pH 8.6 as the separation buffer. Before each run, the capillary is conditioned with NaOH followed by boric acid. Detection is at 260 nm with DAD scans from 200 to 400 nm (Mellenthin and Galensa, 1999).

Soy isoflavones have been separated by CZE and HPLC (Figure 1.16) (Mellenthin and Galensa, 1999). The main advantage of the CZE is the short analysis time (8 min) compared to the 50 min by HPLC. During the CZE separation, the glycosides daidzin and genistin with their higher molecular weight are detected earlier than their aglycones because the negatively charged isoflavone molecules move toward the anode. Daidzin and daidzein are detected before genistin and genistein, respectively, due to charge effects (the extra hydroxy group of genistein tends to lose a proton). However, reproducibility of migration time in CZE is poor. In HPLC, daidzein derivatives elute earlier than those of genistein. The detection limit in HPLC is about 0.01 to 0.03 mg/l compared with 0.1 to 0.5 mg/l in CZE. The HPLC method has superior repeatability, is less dependent on matrix effects, and is more sensitive; therefore, it is more suitable than CZE for the detection of small amounts of analytes. In CZE the daidzein peak and in particular both glycoside peaks in the front part of the electropherogram can be overlapped by other interfering peaks from the matrix. In addition, the moderate reproducibility of migration times in CZE and the narrow detection range of 4 min for eight isoflavones lead to large variation of the results.

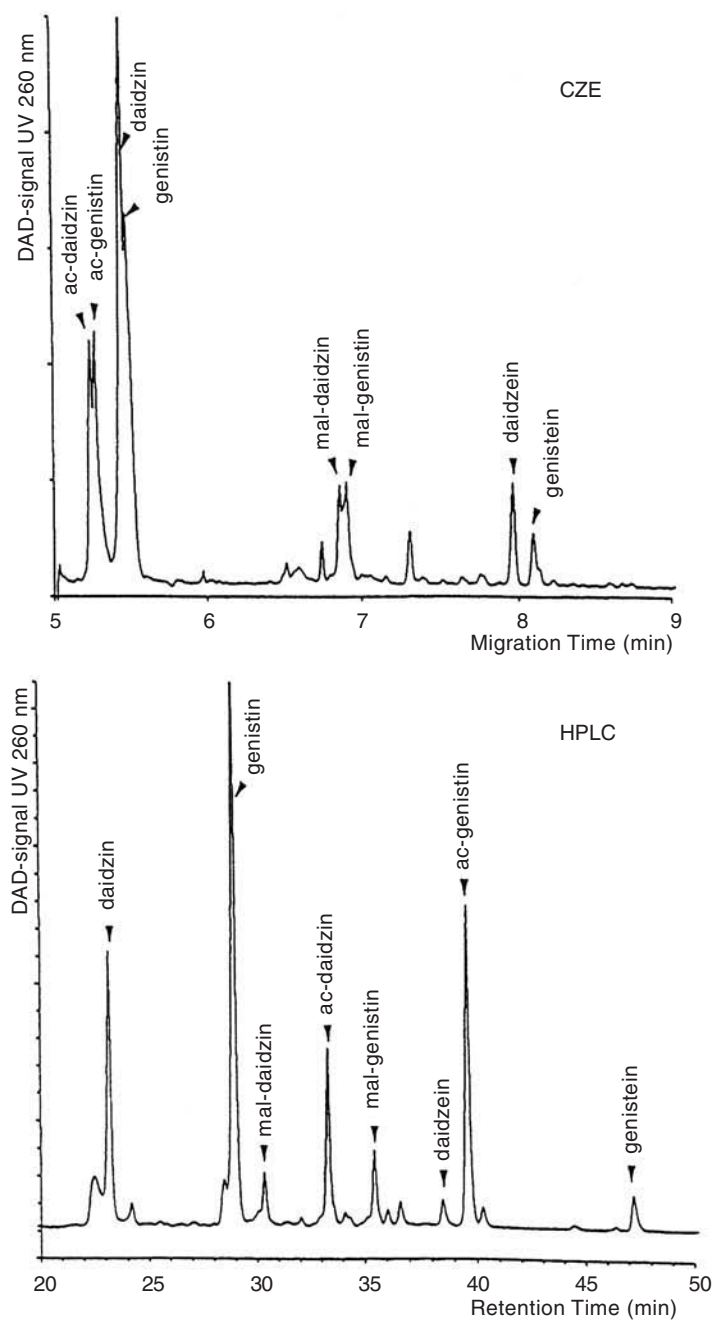


FIGURE 1.16 Separation of isoflavones from toasted soy flour by CZE and HPLC. (Reprinted with permission from Mellenthin, O. and Galensa, R., *J. Agric. Food Chem.*, 47, 594–602, 1999.)

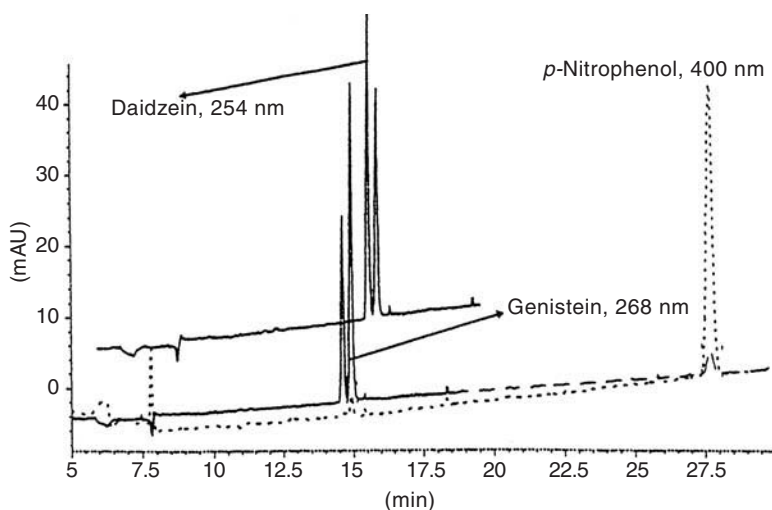


FIGURE 1.17 Electropherogram of a standard mixture of daidzein, genistein, and the internal standard *p*-nitrophenol. (Reprinted with permission from Vääntinen, K. and Moravcova, J., *Czech. J. Food Sci.*, 17, 61–67, 1999.)

1.3.7 HIGH-PERFORMANCE CAPILLARY ELECTROPHORESIS

High-performance capillary electrophoresis (HPCE) is now the most rapidly expanding analytical technique because of its speed and very high separation efficiency, and interest in performing single-step analyses with the simple sample preparation technique in HPCE is considerable. HPCE has been introduced into analytical methods of daidzein and genistein (Aramendia et al., 1995b; Shihabi et al., 1994). Separation by HPCE generally requires a buffer, preferably sodium borate (200 mM) adjusted to pH 8.6; an uncoated capillary (150 μ m I.D. $\times$ 50 cm, 56 cm length); sample injection performed in pressure mode (150 mbars/s), and UV detection set at 254, 268, and 400 nm for daidzein, genistein, and *p*-nitrophenol, respectively. The separation potential at 10 kV corresponding to an electric current of 60 mA, column temperatures of 25°C, and run time of 30 min is used for the extracts. The migration time under these conditions for daidzein and genistein is 13.52 and 13.80 min, respectively (Figure 1.17). The standard deviation of fluctuation of migration time is low, 0.34 min with an RSD of 2.50 and 2.48 for daidzein and genistein, respectively. The resolution and other performance indicators for HPCE of daidzein and genistein are presented in Table 1.13. A major concern of the HPCE method is the instability of the baseline during analysis, resulting in decreasing migration times (Vääntinen and Moravcova, 1999).

1.3.8 MICELLAR ELECTROKINETIC CHROMATOGRAPHY

High separation power such as electrodriven separation methods is quite attractive for the determination of phytoestrogens in biological samples. However, poor concentration

TABLE 1.13
Performance and Noise Determination for HPCE Analysis
of a Standard Mixture (5 mg/l) of Daidzein and Genistein

	Daidzein	Genistein	<i>p</i> -Nitrophenol
Noise (6 × SD) ^a (MAU)	0.1646	0.1648	0.2122
Drift (mAU/h)	3.394	2.947	1.5119
Migration time (min)	13.52	13.87	20.19
Theoretical plate number	93 672	88 241	73 011
Resolution	1.72	1.61	—
S/N (stability/noise ratio)	47.20	57.40	23.1
Limit of detection (mg/dm ³)	0.32	0.26	—
Limit of quantification (mg/dm ³)	1.00	0.90	—

^a Time range from 15 to 20 min.

Source: Vanttinen, K. and Moravcova, J., *Czech. J. Food Sci.*, 17, 61–67, 1999.
 With permission.

detection limits and the influence of interferences from sample constituents are major disadvantages of the method. For these reasons, laser-induced fluorescence (LIF), a technique known for its favorable concentration detection limits, is combined with on-line recording of the fluorescence emission spectra. Micellar electrokinetic chromatography (MEKC) is combined with a special mode of LIF detection for the determination of isoflavonoids and, more especially, formononetin in red clover. In this LIF mode, deep-UV excitation is performed, and fluorescence spectra are recorded on-line. The native fluorescent isoflavonoids are excited at 275 nm by a modified large-frame, argon-ion laser and DAD mounted on a spectrograph. On-line recording of the fluorescence emission spectra simplifies identification of peaks in the electropherogram. Compared to conventional UV absorption detection, interferences are drastically reduced since only sample constituents exhibiting native fluorescence are detected. The identification and quantification of formononetin in a clover extract at a concentration of 3×10^{-5} M or 17 µg formononetin per gram of red clover demonstrate the potential of this approach.

The electrodriven separation of the isoflavonoids is optimized using UV absorbance detection, and complete separation is achieved in 12 min using a micellar system of 50 mM aqueous borate buffers at pH 8.7 containing 50 mM sodium dodecyl sulfate (SDS) (Figure 1.18). Severe conditions are necessary to hydrolyze and extract all phytoestrogens and their conjugates (primarily water-soluble glycosides). After SPE, the sample is diluted fourfold and subsequently analyzed using MEKC. Figure 1.19 shows part of a typical electropherogram — recorded using a new capillary — with prominent peaks at 8.53, 8.97, and 9.62 min (denoted as X, Y, and Z, respectively) in addition to some smaller ones. The repeatability of migration times of a mixture of standards at 5 µM concentration is 1 to 2%. The detection limits for the three fluorescent analytes, daidzein, formononetin, and coumestrol, in

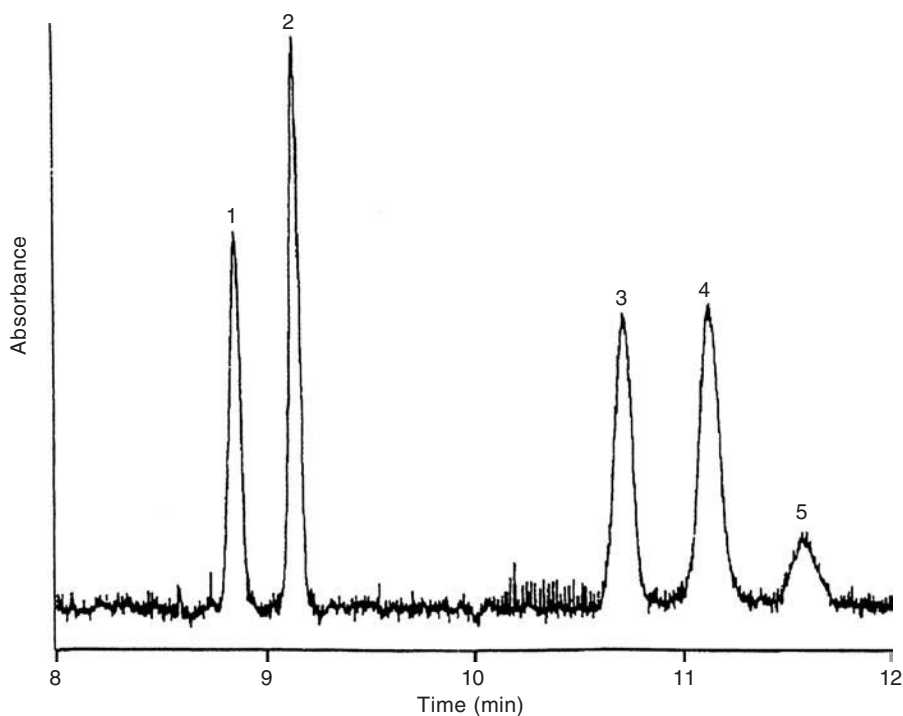


FIGURE 1.18 Electropherogram of an aqueous solution of 5 nM of daidzein (1), genistein (2), formononetin (3), biochanin A (4), and coumestrol (5) using MEKC UV. (From Beekman, M. C., et al., *J. Microcolumn Sep.*, 11, 347–350, 1999. With permission.)

the standard solution at 275 nm LIF detection are between 0.1 and 0.4 μM [signal-to-noise ratio (S/N) 3, where N is peak-to-peak noise]. At analyte concentrations of 1 μM , the RSDs of the peak areas are less than 9% ($n = 5$) (Beekman et al., 1999).

1.3.9 IMMUNOASSAY

Immunoassay can be a powerful alternative to GC-MS- or HPLC-based methods for studying the bioavailability and metabolic fate of dietary isoflavonoids because of its ease, relatively low cost, availability for automation, and suitability for epidemiological studies. GC-MS and HPLC methods are neither suitable for screening purposes in large populations nor sensitive enough for assay of unconjugated phytoestrogens in plasma. Antibodies to some phytoestrogens, including daidzein, have been raised in rabbits as early as 1969 to be used in sheep for passive immunization as protection from an excess of phytoestrogens (Bauminger et al., 1969). The development of sensitive RIA for daidzein, genistein, and formononetin was attempted by Cox et al. (1972), but was never used for quantitative assays. Immunoassays specific for daidzein and its 4'-derivatives (formononetin, 4'-sulfate, and 4'-glucuronide) and for genistein and its 4'-derivatives (biochanin A, 4'-sulfate and 4'-glucuronide of genistein) have recently been developed (Lapcik et al., 1998).

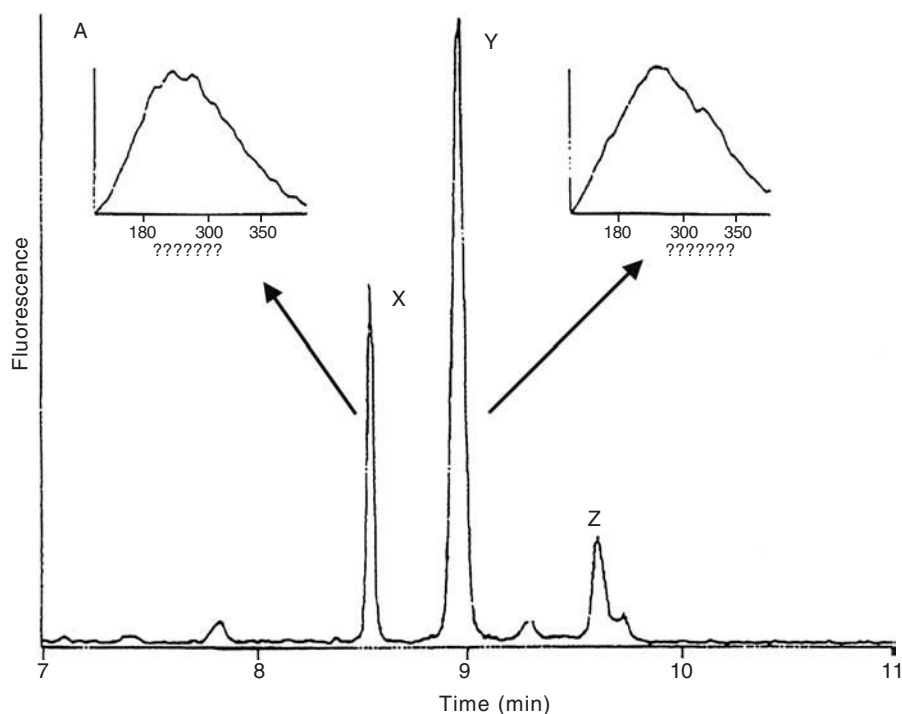


FIGURE 1.19 Electropherogram of clover extract with main peaks indicated as X (formononetin), Y (daidzein), and Z (coumestrol). (From Beekman, M. C., et al., *J. Microcolumn Sep.*, 11, 347–350, 1999. With permission.)

Lapcik et al. (1997) established an RIA for daidzein based on polyclonal antibodies against daidzein-4'-O-(carboxymethyl)ether-BSA. Daidzein is measured in serum plasma, or urine by either direct RIA or RIA following an extraction step with diethyl ether. The dry residue after ether evaporation is dissolved in assay buffer (20 mM sodium phosphate in saline) containing sodium azide and BSA, and an aliquot is measured by RIA. The radioligand and the antibody are dissolved together with the biological fluid in the assay buffer and incubated after vortex mixing; the bound and free portions are separated by dextran-charcoal absorption, and radioactivity is measured in a gamma counter. In the case of direct RIA, the samples of serum or urine are appropriately diluted with the assay buffer in order to obtain the signal within the range of the calibration curve.

The intra- and interassay coefficients of variation, depending on the method (direct or extraction) and concentration of daidzein in the sample, range from 4.1 to 11.5% and from 5.6 to 21.7%, respectively. The assay has low cross-reactivity with other chemically related compounds: 2 to 4% for dihydrodaidzein, 1 to 3% for genistein, 1 to 5% for biochanin A, and 1 to 6% for equol. However, the daidzein values obtained following diethyl ether extraction of human sera is only 8% of that obtained by direct RIA, probably due to the cross-reactivity of daidzein 4'-glucuronides with sulfates present in serum. The RIA method for daidzein is more sensitive

TABLE 1.14
Specificity of RIAs

	Daidzein/Formononetin RIA (% cross-reactivity)	Genistein/Biochanin A RIA (% cross-reactivity)
Daidzein	100	5.5
Formononetin	59.7	3.9
Genistein	1.3	100
Biochanin A	1.5	173
Dihydrodaidzein	2.4	0.36
Equol	1.1	0

Note: Cross-reactivities of selected isoflavonoids and flavonoids in RIA were calculated as the ratio of 50% intercepts of analyte and of potential cross-reactant.

Source: From Lapčík, O. et al., *Steroids*, 63, 14–20, 1998a. With permission.

than previous methods used for quantitation of phytoestrogens in biological fluids. The direct RIA method is well suited for screening and kinetic studies because the signal obtained is proportional to the free analyte after a soy load.

An RIA method has been used to quantitate formononetin in murine plasma and mammary glandular tissue for animal model studies (Wang, 1998). This assay utilizes an antiserum raised in rabbits following immunization with formononetin-7-*O*-(carboxymethyl)ether coupled to bovine serum albumin. The bound and free forms of formononetin are separated by adding dextran-coated charcoal. The RIA procedure enables the quantification of 4 ng/ml of plasma or 50 pg/mg of mammary tissue. The reliability and reproducibility of the assay, demonstrated by intra- and interassay variation, are 6.5 and 11.9%, respectively. The RIA correlates well with a high-performance liquid chromatographic method ($r = 0.980$) in the determination of formononetin extracts of red clover. However, the RIA values are on average 5% higher than those obtained by HPLC, probably due to cross-reactions from other compounds. The mean recovery of formononetin addition in plasma and mammary tissue homogenates is about 87 and 98%, respectively. In practical terms, the assay is efficient since the procedure can readily run 100 samples in a normal working day. Therefore, this RIA, which avoids any sample extraction and preparation, should be suitable for routine monitoring of formononetin in biological fluids or in plants.

The fractions from diethyl ether extracts of beer have been analyzed by RIAs specific for daidzein/formononetin and for genistein/biochanin A (Lapčík et al., 1998a). For this procedure, polyclonal antibodies against conjugates of daidzein-4'-(carboxymethyl)ether and genistein-4'-(carboxymethyl)ether with bovine serum albumin are raised in rabbits. The conjugates of daidzein and genistein are labeled with radioiodinated tyrosine methyl ester for use as radioligands in competitive RIA. The cross-reactivities of the antisera are summarized in Table 1.14. The first immunoassay

is highly specific for daidzein and formononetin; the specificity of the genistein/biochanin A immunoassay is lower with 4 to 5% cross-reaction of daidzein and formononetin. The sensitivity of the assay is 0.08 nmol/l for daidzein and formononetin and 0.15 nmol/l for genistein and biochanin A. The intra- and interassay of this procedure are 5.7 and 9.8% for the daidzein/formononetin and 7.3 and 13.2% for genistein/biochanin A RIAs, respectively (Lapčík et al., 1998a).

RIAs for formononetin in forage, free conjugated daidzein and genistein in plasma, and free isoflavones in beer have also been developed (Lapcik et al., 1997, 1998a,b; Wang et al., 1994). However, the short shelf-life of the labeled compounds has led to the development of time-resolved (TR) FIA that combines the advantages of other nonradioisotopic assays with a 10- to 100-fold increase in sensitivity and assay range in comparison with conventional enzyme immunoassay (EIA) and FIA methods. The first TR-FIA for daidzein in urine and enterolactone in plasma have recently been published (Adlercreutz et al., 1998; Kohen et al., 1998). The latest TR-FIA method uses a europium chelate as the label for the determination of the phytoestrogens daidzein and genistein in plasma for screening and kinetic studies (Wang et al., 2000b). In this procedure, 4'-*O*-carboxymethyl derivatives of daidzein and genistein are synthesized, coupled to bovine serum albumin, and used as antigens to immunize rabbits. Immunoassay is carried out after enzymatic hydrolysis and ether extraction. The method is highly sensitive, with a detection limit of 1.8 and 3.1 pg/20 µl for daidzein and genistein, respectively, and precise, with intra- and interassay coefficients of variation (CV%) between 3.2 and 6.3%. Although the antisera cross reacts with some isoflavonoids, values obtained by the TR-FIA method correlate highly ($r \geq 0.95$) with those based on ID-GC-MS. The advantages of this technology have been described as being highly sensitive, low background interference, wide dynamic range, reliable, relatively inexpensive, and practical with short incubation time and completion of 96 samples in 4 h (Wang et al., 2000b). These advantages enable the TR-FIA method to be well established in routine immunodiagnostic work.

1.3.10 BIOASSAY

In the age of genomics, bioassays using reporter genes for the detection or quantitative evaluation of estrogen are becoming commonplace. Phytoestrogens through their estrogenic effect are known to regulate transcription of specific genes via estrogen receptors (Shiizaki et al., 1999). A highly sensitive bioassay system has been developed by placing estrogen-responsive elements upstream to the reporter gene, and this assay has been used to determine the estrogen activity in Chinese herbal prescriptions used for postmenopausal disorder (Shiizaki et al., 1999). For this procedure, plasmids are constructed by inserting sequences of estrogen-responsive elements upstream to the luciferase gene, stable transfection of these plasmids into MCF-7 human cancer cells are obtained, and cell strains with luciferase activity are then used to evaluate the herbal prescriptions. In contrast to immunoassay, the bioassay is independent of the structures of active substances, is more sensitive, and is considered highly suitable for assessing phytoestrogens.

1.4 CONCLUSIONS

A wide range of analytical techniques are available for the detection, quantitation, identification, evaluation, and monitoring of phytoestrogens in biological materials. The methods vary from simple to complex depending on extraction, separation, fractionation, identification, and detection of the analytes. HPLC has become the method of choice for the evaluation and determination of phytoestrogens, especially from plant sources, because of its widespread availability, affordability, and adequate sensitivity. The development of atmospheric pressure ion sources such as the ES and APCI has added LC-MS to the list of bioanalytical assays providing higher specificity, precision, accuracy, sensitivity, applicability, and a wider dynamic range than HPLC. Notwithstanding the sudden surge in the analytic capability of the separation and identification of phytoestrogens on the basis of their molecular structure, the HPLC-MS method is not quite the standard yet. However, the ability of HPLC-MS to characterize and quantify the full range of phytoestrogens can help in accurately assessing dietary intake of phytoestrogens that may interfere with epidemiological attempts to elicit a relationship between phytoestrogens and health. To date, only the complicated ID-GC-MS-SIM method can determine phytoestrogens from the three main groups: isoflavones, lignans, and coumestans. Methods such as CE with LIF detection have the ability to detect the distribution, localization, and diversity of phytoestrogens in individual cells and organelles and may lead to early diagnosis of phytoestrogen-deficient diseases. It is therefore necessary to rethink and reevaluate new analytical techniques so that all phytoestrogens can be detected and identified simply and reliably.

REFERENCES

- Adlercreutz, H., van der Wildt, J., Attala, H., Wähälä, K., Mäkelä, T., Hase, T., and Fotsis, T., Lignan and Isoflavonoid conjugates in human urine, *J. Steroid Biochem. Mol. Biol.*, 52, 97–103, 1995.
- Adlercreutz, H., Western diet and Western diseases: some hormonal and biochemical mechanisms and associations, *Scand. J. Clin. Invest. Suppl.*, 201, 3, 1990.
- Adlercreutz, H., Phytoestrogens: epidemiology and a possible role in cancer protection, *Environ. Health Perspect.*, 103, 103–112, 1995.
- Adlercreutz, H., Phytoestrogens. State of the art, *Environ. Toxicol. Pharmacol.*, 7, 201–207, 1999.
- Adlercreutz, H. and Mazur, W., Phyto-oestrogens and Western diseases, *Ann. Med.*, 29, 95–120, 1997.
- Adlercreutz, H., Fotsis, T., Bannwart, C., Wähälä, K., Mäkelä, T., Brunow, G., and Hase, T., Determination of urinary lignans and phytoestrogen metabolites, potential antiestrogens, and anticarcinogens, in urine of women on various habitual diets, *J. Steroid Biochem. Mol. Biol.*, 25, 791–797, 1986.
- Adlercreutz, H., Honjo, H., Higashi, A., Fotsis, T., Hämäläinen, E., Hasegawa, T., Okada, H., Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet, *Am. J. Clin. Nutr.*, 54, 1093–1100, 1991a.

- Adlercreutz, H., Fotsis, T., Banwart, C., Wähälä, K., Brunow, G., and Hase, T., Isotope dilution gas chromatographic-mass spectrometric method for the determination of lignans and isoflavonoids in human urine including identification of genistein, *Clin. Chim. Acta*, 199, 263–278, 1991b.
- Adlercreutz, H., Fotsis, T., Lampke, J., Wähälä, K., Mäkelä, T., Brunow, G., and Hase, T., Quantitative determination of lignans and isoflavonoids in plasma of omnivorous and vegetarian women by isotope dilution gas chromatography-mass spectrometry, *Scand. J. Clin. Lab. Invest.*, 53, 5–18, 1993.
- Adlercreutz, H., Fotsis, T., Watanabe, S., Lampe, J., Wähälä, K., Mäkelä, T., and Hase, T., Determinations of lignans and isoflavonoids in plasma by isotope dilution gas chromatography-mass spectrometry, *Cancer Prev.*, 18, 259–1271, 1994.
- Adlercreutz, H., Fotsis, T., Kurzer, M.S., Wähälä, K., Mäkelä, T., and Hase, T., Isotope dilution gas chromatographic-mass spectrometric method for the determination of lignans and isoflavonoids in human feces, with preliminary results in omnivorous and vegetarian women, *Anal. Biochem.*, 225, 101–108, 1995.
- Adlercreutz, H., Wang, G.J., Lapčák, O., Hampl, R., Wähälä, K., Mäkelä, T., Lusa, K., Talme, M., and Mikola, H. Time-resolved fluoroimmunoassay for plasma enterolactone, *Anal. Biochem.*, 265, 208–215, 1998.
- Aramendia, M.A., Garcia, I., Lafont, F., and Marinas, J.M., Determination of isoflavones using high-performance liquid chromatography with atmospheric-pressure chemical ionization mass spectrometry, *Rapid Commun. Mass Spectrom.*, 9, 503–508, 1994.
- Aramendia, M.A., Garcia, I., Lafont, F., and Marinas, J.M., Determination of isoflavones using capillary electrophoresis in combination with electrospray mass spectrometry, *J. Chromatogr.*, 707, 327–333, 1995a.
- Aramendia, M.A., Borau, V., Garcia, I., Jimenez, C., Lafont, F., Marinas, J.M., Porras, A., and Urbano, F.J., Determination of isoflavones by capillary electrophoresis/electrospray ionization mass spectrometry, *J. Mass Spectrom. Rapid Commun. Mass Spectrom.*, 23 S153–S157, 1995b.
- Atkinson, D.A., Hill, H.H., and Shultz, T.D., Quantification of mammalian lignans in biological fluids using gas chromatography with ion mobility detection, *J. Chromatogr. Biomed. Appl.*, 617, 173–179, 1993.
- Bakke, J.E. and Klosterman, H.T., A new diglucoside from flaxseed, *Proc. N.D. Acad. Sci.*, 10, 18–22, 1956.
- Bambagiotti-Alberti, M., Coron, S.A., Ghiara, C., Giannellini, V., and Raffaelli, A., Revealing the mammalian lignan precursor secoisolariciresinol diglucoside in flaxseed by ion-spray mass spectrometry, *Rapid Commun. Mass Spectrom.*, 8, 595–598, 1994a.
- Bambagiotti-Alberti, M., Coron, S.A., Ghiara, C., Moneti, G., and Raffaelli, A., Investigation of mammalian lignan precursors in flaxseed: first evidence of secoisolariciresinol diglucoside in two isomeric forms of liquid chromatography/mass spectrometry, *Rapid Commun. Mass Spectrom.*, 8, 929–932, 1994b.
- Barbuch, R.J., Coutant, J.E., Welsh, M.B., and Setchell, K.D.R., The use of thermospray liquid chromatography/tandem mass spectrometry for the class identification and structural verification of phytoestrogens in soy protein preparations, *Biomed. Environ. Mass Spectrom.*, 18, 973–977, 1989.
- Barnes, B., Kirk, M., and Coward, L., Isoflavones and their conjugates in soy foods: extraction and analysis by HPLC-mass spectrometry, *J. Agric. Food Chem.*, 42, 2466–2474, 1994.
- Barnes, K.A., Smith, R.A., Williams, K., Damant, A.P., and Shepherd, M.J., A microbore high performance liquid chromatography/electrospray ionization mass spectrometry method for the determination of the phytoestrogens genistein and daidzein in comminuted baby foods and soya flour, *Rapid Commun. Mass Spectrom.*, 12, 130–138, 1998.

- Barnes, S., Coward, L., Kirk, M., and Sfakianos, J., HPLC-mass spectrometry analysis isoflavones, *Proc. Soc. Exp. Biol. Med.*, 217, 254–262, 1998.
- Bauminger, S., Lindner, H.R., Perel, E., and Arnon, R., Antibodies to a phyto-oestrogen: antigenicity of genistein coupled to a synthetic polypeptide, *J. Endocrinol.*, 44, 567–578, 1969.
- Beekman, M.C., Lingeman, H., Brinkman, U.A. Th., and Gooijer, C., Determination of the isoflavone formononetin in red clover (*Trifolium pratense* L.) by micellar electrokinetic chromatography combined with deep-UV laser-induced wavelength-resolved fluorescence detection, *J. Microcolumn Sep.*, 11, 347–350, 1999.
- Bickoff, E.M., Loper, G.M., Hanson, C.H., Graham, J.H., Witt, S.C., and Spencer, R.R., Effect of common leafspot on coumestans and flavones in alfalfa, *Crop Sci.*, 7, 259–261, 1967.
- Bingham, S.A., Atkinson, C., Liggins, J., Bluck, L., and Coward, A., Phyto-oestrogens: where are we now? *Br. J. Nutr.*, 79, 393–406, 1998.
- Brandi, M.L., Phytoestrogens and menopause, *Environ. Toxicol. Pharmacol.*, 7, 213–216, 1999.
- Brzezinski, A. and Debi, A., Phytoestrogens: the “natural” selective estrogens receptor modulators? *Eur. J. Obstet. Gynecol.*, 85, 47–51, 1999.
- Bylund, A., Zhang, J.-X., Bergh, A., Damber, J.-E., Widmark, A., Johansson, A., Adlercreutz, H., Aman, P., Shepherd, M.J., and Hallmans, G., Rye bran and soy protein delay growth and increase apoptosis of human LNCaP prostate adenocarcinoma in nude mice, *Prostate*, 42, 304–314, 2000.
- Careri, M., Mangia, A., and Musci, M., Overview of the applications of liquid chromatography-mass spectrometry interfacing systems in food analysis: naturally occurring substances in food, *J. Chromatogr. A*, 794, 263–297, 1998.
- Carlson, R.E. and Dolphin, D., High-performance liquid chromatographic method for the analysis of isoflavones, *J. Chromatogr.*, 198, 193–197, 1980.
- Carusi, D., Phytoestrogens as hormone replacement therapy: an evidence-based approach, *Prim. Care Update Obstet. Gynecol.*, 7, 253–259, 2000.
- Cassidy, A., Bingham, S., and Setchell, K.D.R., Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women, *Am J. Clin. Nutr.*, 60, 333–340, 1994.
- Chandra, A. and Nair, M.G., Supercritical carbon dioxide extraction of daidzein and genistein from soybean products, *Phytochem. Anal.*, 7, 259–262, 1996.
- Chansakaow, S., Ishikawa, T., Seki, H., Sekine, K., Okada, M., and Chaichantipyuth, C., Identification of deoxymiroestrol as the actual rejuvenating principle of “Kwao Keur,” *Pueraria mirifica*. The known mirosterol may be an artifact, *J. Nat. Prod.*, 63, 173–175, 2000.
- Coward, L., Barnes, N.C., Setchell, K.D.R., and Barnes, S., The antitumor isoflavones, genistein and daidzein, in soybean foods of American and Asian diets, *J. Agric. Food Chem.*, 41, 1961–1967, 1993.
- Coward, L., Kirk, M., Albin, N., and Barnes, S., Analysis of plasma isoflavones in the reversed-phase HPLC-multiple reaction ion monitoring-mass spectrometry, *Clin. Chim. Acta*, 247, 121–142, 1996.
- Cox, R.I. and Davies, L.H., Modification of pasture oestrogens in the gastrointestinal tract of ruminants, *Proc. Nutr. Soc. Aust.*, 13, 61–67, 1988.
- Cox, R.I., Wong, M.S., Braden, A.W., Trikojus, V.M., and Lindner, H.R., The formation and specificity of antibodies to phyto-oestrogens in the sheep, *J. Reprod. Fertil.*, 28, 157–158, 1972.
- Davin, B.L. and Lewis, N.G., Dirigent proteins and dirigent sites explain the mystery of specificity of radical precursor coupling in lignan and lignan biosynthesis, *Plant Physiol.*, 123, 453–461, 2000.

- Davin, B.L., Wang, H.B., Crowell, A.L., Bedgar, D.L., Martin, D.M., Sarkanen, S., and Lewis, N.G., Stereoselective biomolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center, *Science*, 75, 362–366, 1997.
- Decennia, L., Roughstock, A., Gantt, M., and Thibier, M., Identification and quantitative estimation of a lignan in human and bovine semen, *J. Reprod. Fertil.*, 66, 305–309, 1982.
- Dewald, H.D., Worst, S.A., Butcher, J.A., and Saulinskas, E.F., Separation and identification of isoflavones with online liquid chromatography — electrochemistry thermospray mass spectrometry, *Electroanalysis*, 3, 777–782, 1991.
- Dixon, R.A., Isoflavonoids: biochemistry, molecular biology, and biological functions, in *Comprehensive Natural Products Chemistry*, Vol. 1, Sankawa, U, Ed., Elsevier, Amsterdam, 1999, pp. 773–823.
- Dixon, R.A. and Pavia, N.L., Stress-induced phenylpropanoid metabolism, *Plant Cell*, 7, 1085–1097, 1995.
- Dixon, R.A. and Steele, C.L., Flavonoids and isoflavonoids — a gold mine for metabolic engineering, *Trends Plant Sci.*, 4, 394–400, 1999.
- Dziedzic, S.Z. and Dick, J., Analysis of isoflavones in bengalgram by high-performance liquid chromatography, *J. Chromatogr.*, 234, 497–499, 1982.
- Eldridge, A.C., Determination of isoflavones in soybean flours, protein concentrates, and isolates, *J. Agric. Food Chem.*, 30, 353–355, 1982a.
- Eldridge, A.C., High-performance liquid chromatography separation of soybean isoflavones and their glucosides, *J. Chromatogr.*, 234, 494–496, 1982b.
- Eldridge, A.C. and Kwolek, W.F., Soybean isoflavones: effect of environment and variety on composition, *J. Agric. Food Chem.*, 31, 394–396, 1983.
- Farmakalidis, E. and Murphy, P.A. Oestrogenic response of the CD-1 mouse to the soya-bean isoflavones genistein, genistin and daidzin, *Food Chem. Toxicol.*, 22, 237–239, 1984.
- Farnsworth, N.R., Bingel, A.S., Cordell, G.A., Crane, F.A., and Fond, H.S.S., Potential value of plants as source of new antifertility agents, *J. Pharmacol. Sci.*, 64, 717–754, 1975.
- Federal Register, Food labeling: health claims; soy protein and coronary heart disease. Food and Drug Administration, HHS. Final rule, *Fed. Regist.*, 64, 57700–57733, 1999.
- Ford, T.D., Davin, L.B., and Lewis, N.G., Chemopreventive flaxseed lignans: toward bioengineering the metabolic pathways, Proceedings of the 58th Flax Institute of the United States, Fargo, ND, March 23–25, 2000, pp. 39–48.
- Fotsis, T., Heikkinen, R., Adlercreutz, H., Axelson, M., and Setchell, K.D., Capillary gas chromatographic method for the analysis of lignans in human urine, *Clin. Chim. Acta*, 121, 361–371, 1982.
- Franke, A.A., Custer, L.J., Cerna, C.M., and Narala, K.K., Quantitation of phytoestrogens in legumes by HPLC, *J. Agric. Food Chem.*, 42, 1905–1913, 1994.
- Franke, A.A., Custer, L.J., Cerna, C.M., and Narala, K.K., Rapid HPLC analysis of dietary phytoestrogens from legumes and from human urine, *Proc. Soc. Exp. Biol. Med.*, 208, 18–26, 1995.
- Franke, A.A. and Custer, L.J., Daidzein and genistein concentrations in human milk after soy consumption, *Clin. Chem.*, 42, 955–964, 1996.
- Franke, A.A., Custer, L.J., Wang, W., and Shi, C.Y., HPLC analysis of isoflavonoids and other phenolic agents from foods and from human fluids, *Proc. Soc. Exp. Biol. Med.*, 217, 263–273, 1998.
- Franke, S.S. and Custer, L.J., High-performance liquid chromatographic assay of isoflavonoids and coumestrol from human urine, *J. Chromatogr.*, 662, 47–60, 1994.
- Fritz, W.A., Coward, L., Wang, J., and Lamartiniere, C.A., Dietary genistein: perinatal mammary cancer prevention, bioavailability and toxicity testing in the rat, *Carcinogenesis*, 19, 2151–2158, 1998.

- Gamache, P., Ryan, E., and Acworth, I.N., Analysis of phenolic and flavonoid compounds in juice beverages using high-performance liquid chromatography with coulometric array detection, *J. Chromatogr.*, 635, 143–150, 1993.
- Gamache, P.H. and Acworth, I.N., Analysis of phytoestrogens and polyphenols in plasma, tissue, and urine using HPLC with coulometric array detection, *Proc. Soc. Exp. Biol. Med.*, 217, 274–280, 1998.
- Garrett, S.D., Lee, H.A., Friar, P.M.K., and Morgan, M.R.A., Validation of a novel estrogen receptor-based microtitration plate assay for the determination of phytoestrogens in soy-based foods, *J. Agric. Food Chem.*, 47, 4106–4111, 1999.
- Gavaler, J.S., Rosenblum, E.R., Deal, S.R., and Bowie, B.T., The phytoestrogen congeners of alcoholic beverages: current status, *Proc. Soc. Exp. Biol. Med.*, 208, 98–102, 1995.
- Gennari, C., Agnusdei, D., Crepaldi, G., Isaia, G., Mazzuoli, G., Ortolani, S., Bufalino, L., and Passeri, M., Effect of ipriflavone-a synthetic derivative of natural isoflavones on bone mass loss in the early years after menopause, *Menopause*, 5, 9–15, 1998.
- Graham, T.L., A rapid high-resolution high performance liquid chromatography profiling procedure for plant and microbial aromatic secondary metabolites, *Plant Physiol.*, 95, 584–593, 1991a.
- Graham, T.L., Flavonoid and isoflavonoid distribution in developing soybean seedling tissue and in seed and root exudates, *Plant Physiol.*, 95, 594–603, 1991b.
- Graham, T.L., King, J.E., and Graham, M.Y., Role of constitutive isoflavone conjugates in the accumulation of glyceollin in soybean infected with *Phytophthora megasperma*, *Mol. Plant-Microbe Interact.*, 3, 157–166, 1990.
- Hampl, R., Lapčík, O., Wähälä, K., Starka, L., and Adlercreutz, H., Radioimmunoanalyza fytoestrogenů isoflavonoidovými, *Chem. Listy*, 92, 44–50, 1998.
- Harris, R.K., Greaves, J., Alexander, D., Wilson, T., and Haggerty, W.J., Development of stability-indicating analytical methods for flaxseed lignans and their precursors, in *Food Phytochemicals II: Teas, Spices, and Herbs*, Osawa, C.-T., Huang, M.-T., and Rosen, R.T., Eds., American Chemical Society, Washington, D.C., 1994, pp. 295–305.
- Hutabarat, L.S., Mulholland, M., and Greenfield, H., Development and validation of an isocratic high-performance liquid chromatographic method for quantitative determination of phytoestrogens in soya bean, *J. Chromatogr.*, 795, 377–382, 1998.
- Hutchins, A.M., Slavin, J.L., and Lampe, J.W., Urinary isoflavonoid phytoestrogen and lignan excretion after consumption of fermented and unfermented soy products, *J. Am. Diet. Assoc.*, 95, 545–551, 1995.
- Hutchins, A.M., Lampe, J.W., Martini, M.C., Campbell, D.R., and Slavin, J.L., Vegetables, fruits and legumes: effect on urinary isoflavonoid phytoestrogen and lignan excretion, *J. Am. Diet. Assoc.*, 95, 769–774, 1995.
- Ingram, D., Sanders, K., Kolybaba, M., and Lopez, D., Case-control study of phytoestrogens and breast cancer, *Lancet*, 350, 990–994, 1997.
- Ishimi, Y., Miyauchi, C., Ohmura, M., Onoe, Y., Sato, T., Uchiyama, Y., Ito, M., Wang, X., Suda, T., and Ikegami, S., Selective effects of genistein, a soybean isoflavone, on B-lymphopoiesis and bone loss caused by estrogen deficiency, *Endocrinology*, 140, 1893–1900, 1999.
- Joannou, G.E., Kelly, G.E., Reeder, A.Y., Waring, M., and Nelson, C., A urinary profile study of dietary phytoestrogens: the identification and mode of metabolism of new isoflavonoids, *J. Steroid Biochem. Mol. Biol.*, 54, 167–184, 1995.
- Johnson, P., Kamal-Eldin, A., Lundgren, L.N., and Aman, P., HPLC method for analysis of secoisolariciresinol diglucoside in flaxseeds, *J. Agric. Food Chem.*, 48, 5216–5219, 2000.

- Jones, A.E., Price, K.R., and Fenwick, G.R., Development and application of a high-performance liquid chromatographic method for the analysis of phytoestrogens, *J. Sci. Food Agric.*, 46, 357–364, 1989.
- Jorgensen, K., Suter, D.A.I., Thompson, W.K., Dalais, F.S., Rice, G.E., and Wahlqvist, M.L., Burgen® Soy-Lin™. Development of an innovative functional staple food, *Food Aust.*, 50, 297–299, 1998.
- Jung, W., Yu, O., Lau, S.-M.C., O'Keefe, D.P., Odell, J., Fader, G., and McGonigle, B., Identification and expression of isoflavone synthase, the key enzyme for biosynthesis in legumes, *Nat. Biotechnol.*, 18, 208–212, 2000.
- Karas, M., Matric-assisted laser desorption ionization MS: a progress report, *Biochem. Mass Spectrom.*, 24, 897–900, 1996.
- Kardinaal, A.F.M., Waalkens-Berendsen, D.H., and Arts, C.J.M., Pseudo-oestrogens in the diet: health benefits and safety concerns, *Trends Food Sci. Technol.*, 8, 327–333, 1997.
- Kelly, G.E., Nelson, C., Waring, M.A., Joannou, G.E., and Reeder, A.Y., Metabolites of dietary (soya) isoflavones in human urine, *Clin. Chim. Acta*, 223, 9–22, 1993.
- Kinoshita, E., Ozawa, Y., and Aishima, T., Novel tartaric acid isoflavone derivatives that play key roles in differentiating Japanese soy sauces, *J. Agric. Food Chem.*, 45, 3753–3759, 1997.
- Kinoshita, E., Sugimoto, T., Ozawa, Y., and Aishima, T., Differentiation of soy sauce produced from whole soybeans and defatted soybeans by pattern recognition analysis of HPLC profiles, *J. Agric. Food Chem.*, 46, 877–883, 1998.
- Kirkman, L.M., Lampe, J.W., Campbell, D.R., Martini, M.C., and Slavin, J.L., Urinary lignan and isoflavonoid excretion in men and women consuming vegetable and soy diets, *Nutr. Cancer*, 24, 1–12, 1995.
- Kitada, Y., Ueda, Y., Yamamota, M., Ishikawa, M., Nakazawa, H., and Fujita, M., Determination of isoflavones in soy bean by high-performance liquid chromatography with amperometric detection, *J. Chromatogr.*, 366, 403–406, 1986.
- Klejdus, B., Vitamvasova, D., and Kuban, V., Reversed-phase high performance liquid chromatographic determination of isoflavones in plant materials after isolation by solid-phase extraction, *J. Chromatogr. A*, 839, 261–263, 1999.
- Knight, D.C., Eden, J.A., Huang, J.L., and Waring, M.A., Isoflavone content of infant foods and formulas, *J. Pediatr. Child Health*, 34, 135–138, 1998.
- Knuckles, B.E., deFremery, D., and Kohler, G.O., Coumestrol content of fractions obtained during wet processing of alfalfa, *J. Agric. Food Chem.*, 24, 1177–1180, 1976.
- Kohen, F., Lichter, S., Gayer, B., Deboever, J., and Lu, L., The measurement of the isoflavone daidzein by time resolved fluorescent immunoassay: a method of assessment of dietary soya exposure, *J. Steroid Biochem. Mol. Biol.*, 64, 217–222, 1998.
- Kopp, P., Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the 'French paradox'? *Eur. J. Endocrinol.*, 138, 619–620, 1998.
- Koupai-Abyazani, M.R., Creaser, C.S., and Stephenson, G.R., Separation and identification of flavone, flavonol, isoflavone and flavanone aglycones by capillary column gas chromatography, *Phytochem. Anal.*, 3, 80–84, 1992.
- Kudou, S., Fleury, Y., Welti, D., Magnolato, D., Uchida, T., Kitamura, K., and Okubo, K., Malonyl isoflavone glycosides in soybean seeds (*Glycine max* Merrill), *Agric. Biol. Chem.*, 55, 2227–2233, 1991.
- Kurzer, M.S. and Xu, X., Dietary phytoestrogens, *Annu. Rev. Nutr.*, 17, 353–381, 1997.
- Kurzer, M.S., Lampe, J.W., Martini, M.C., and Adlercreutz, H., Fecal lignan and isoflavonoid excretion in premenopausal women consuming flaxseed powder, *Cancer. Epidemiol. Biomarkers Prev.*, 4, 353–358, 1995.

- Lapčák, O., Hampl, R., Al-Maharik, N., Salakka, A., Wähälä, K., and Adlercreutz, H., A novel radioimmunoassay for daidzein, *Steroids*, 62, 315–320, 1997.
- Lapčák, O., Hill, M., Hampl, R., Wähälä, K., Adlercreutz, H., Identification of isoflavonoids in beer, *Steroids*, 63, 14–20, 1998a.
- Lapčák, O., Hampl, R., Hill, M., Wähälä, K., Al-Maharik, N., Adlercreutz, H., Radioimmunoassay of free genistein in human serum, *J. Steroid Biochem. Mol. Biol.*, 64, 261–268, 1998b.
- Lee, H.S., HPLC analysis of phenolic compounds, in *Food Analysis by HPLC*, Nollet, L.M.L., Ed., Marcel Dekker, New York, 2000, pp. 775–824.
- Liggins, J., Bluck, L.J.C., Coward, W.A., and Bingham, S.A., Extraction and quantification of daidzein and genistein in food, *Anal. Biochem.*, 264, 1–7, 1998.
- Liggins, J., Grimwood, R., and Bingham, S.A., Extraction and quantification of lignan phytoestrogens in food and human samples, *Anal. Biochem.*, 287, 102–109, 2000.
- Lu, R. and Serrero, G., Resveratrol, a natural product derived from grape, exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells, *J. Cell. Physiol.*, 179, 297–304, 1999.
- Lundh, T.J.O., Pettersson, J., and Kiessling, K.H., Liquid chromatographic determination of the phytoestrogens daidzein, formononetin, coumestrol, and equol in bovine blood plasma and urine, *J. Assoc. Off. Anal. Chem.*, 71, 938–941, 1988.
- Markham, K.R., *Techniques of Flavonoid Identification*, Academic Press, New York, 1982, p. 113.
- Mazur, W., Phytoestrogen content in foods, *Baillière's Clin. Endocrinol. Metab.*, 12(4), 729–742, 1998.
- Mazur, W.M. and Adlercreutz, H., Naturally occurring oestrogens in food, *J. Pure Appl. Chem.*, 70, 1759–1776, 1998.
- Mazur, W., Fotsis, T., Wähälä, K., Ojala, S., Salakka, A., and Adlercreutz, H., Isotope dilution gas chromatographic-mass spectrometric method for the determination of isoflavonoids, coumestrol, and lignans in food samples, *Anal. Biochem.*, 233, 169–180, 1996.
- Mazur, W.M., Wähälä, K., Ojala, S., Makkonen, A., Hase, T., and Adlercreutz, H., Lignan and isoflavonoid polyphenols in tea and coffee, *Br. J. Nutr.*, 79, 37–45, 1998.
- Meagher, L.P., Beecher, G.R., Flanagan, V.P., and Li, B.W., Isolation and characterization of the lignans, isolariciresinol and pinoresinol in flaxseed meal, *J. Agric. Food Chem.*, 47, 3173–3180, 1999.
- Mellenthin, O. and Galensa, R., Sojanachweis in fleischprodukten mittels kapillarzonenelektrophorese und diodenarraydetektion, *Dtsch. Lebensm. Rundsch.*, 92, 171–175, 1996.
- Mellenthin, O. and Galensa, R., Nachweis von soja in fleischprodukten durch diodenarraydetektion ausgewählter isoflavonoide: vergleich von CZE und HPLC, *Lebensmittelchemie*, 51, 23, 1997.
- Mellenthin, O. and Galensa, R., Analysis of phenols using capillary zone electrophoresis and HPLC: detection of soy, lupin and pea protein in meat products, *J. Agric. Food Chem.*, 47, 594–602, 1999.
- Merken, H.M. and Beecher, G.R., Measurement of food flavonoids by high-performance liquid chromatography: a review, *J. Agric. Food Chem.*, 48, 577–599, 2000.
- Messina, M.J., Persky, V., Setchell, K.D.R., and Barnes, S., Soy intake and cancer risk: a review of the *in vitro* and *in vivo* data, *Nutr. Cancer*, 21, 113–131, 1994.
- Milligan, S.R., Kalita, J.C., Heyerick, A., Rong, H., De Cooman, L., and De Keukeleire, D., Identification of a potent phytoestrogen in hops (*Humulus lupulus* L.) and beer, *J. Clin. Endocrinol. Metab.*, 83, 2249–2252, 1999.

- Morton, M.S., Wilcox, G., Wahlqvist, M.L., and Griffiths, K., Determination of lignans and isoflavonoids in human female plasma following dietary supplementation, *J. Endocrinol.*, 142, 251–259, 1994.
- Morton, M.S., Matos-Ferreira, A., Abrances-Monteiro, L., Coreia, R., Blacklock, N., Chan, P.S.F., Cheng, C., Lloyds, S., Chieh-Ping, W., and Griffiths, K., Measurement and metabolism of isoflavonoids and lignans in the human male, *Cancer Lett.*, 114, 145–151, 1997.
- Morton, M.S., Arisaka, O., Miyake, A., and Evans, B., Analysis of phyto-estrogens by gas-chromatography-mass spectrometry, *Environ. Toxicol. Pharmacol.*, 7, 221–225, 1999.
- Muir, A.D., Westcott, H.D., Ballantyne, K., and Northrup, S., Flax lignans — recent developments in the analysis of lignans in plant and animal tissue, Proceedings of the 58th Flax Institute of the United States, Fargo, ND, 2000, pp. 23–32.
- Müllner, C. and Sontag, G., Determination of some phytoestrogens in soybeans and their processed products with HPLC and coulometric electrode array detection, *Fresenius Z. Anal. Chem.*, 364, 261–265, 1999.
- Murkies, A.L., Wilcox, G., and Davis, S.R., Phytoestrogens, *J. Clin. Endocrinol. Metab.*, 83, 297–303, 1998.
- Murphy, P.A., Separation of genistin, daidzein and their aglucones, and coumestrol by gradient high-performance liquid chromatography, *J. Chromatogr.*, 211, 166–169, 1981.
- Murphy, P.A., Phytoestrogen content of processed soybean products, *Food Technol.*, 36, 62–64, 1982.
- Murphy, P.A., Song, T., Buseman, G., and Barua, K., Isoflavones in soy-based infant formulas, *J. Sci. Food Agric.*, 45, 4635–4638, 1997.
- Nestel, P.J., Pomeroy, S., Kay, S., Komesaroff, P., Behrsing, J., Cameron, J.D., and West, L., Isoflavones from red clover improve systemic arterial compliance but not plasma lipids in menopausal women, *J. Clin. Endocrinol. Metab.*, 84, 895–898, 1999.
- Nicollier, G.F., Separation and quantitation of estrogenic isoflavones from clovers by high-performance liquid chromatography, *J. Chromatogr.*, 249, 399–402, 1982.
- Nurmi, T. and Adlercreutz, H., Sensitive HPLC method for profiling phytoestrogens using coulometric electrode array detection: application to plasma analysis, *Anal. Biochem.*, 274, 110–117, 1999.
- Obermeyer, W.R., Musser, S.M., Betz, J.M., Casey, R.E., Pohland, A.E., and Page, S.W., Chemical studies of phytoestrogens and related compounds in dietary supplements: flax and chaparral, *Proc. Soc. Exp. Biol. Med.*, 208, 6–12, 1995.
- Ohta, N., Kuwata, G., Akahori, H., and Watanabe, T., Isoflavonoid constituents of soybeans and isolation of a new acetyl daidzin, *Agric. Biol. Chem.*, 43, 1415–1419, 1979.
- Okubo, K., Fijima, M., Kobayashi, Y., Yoshikoshi, M., Uchida, T., and Kudou, S., Components responsible for the undesirable taste of soybean seeds, *Biosci. Biotechnol. Biochem.*, 56, 99–103, 1992.
- Oomah, B.D. and Mazza, G., Functional foods, in *The Wiley Encyclopedia of Science and Technology*, 2nd ed., Vol. 2, Francis, F.J., Ed., Wiley, New York, 2000, pp. 1176–1182.
- Payne, T.J., Promoting better health with flaxseed in bread, *Cereal Food World*, 45, 102–104, 2000.
- Padmavati, M. and Reddy, A.R., Flavonoid biosynthetic pathway and cereal defence response: an emerging trend in crop biotechnology, *Plant Biochem. Biotechnol.*, 8, 15–20, 1999.
- Peterson, G. and Barnes, S., Genistein inhibition of the growth of human breast cancer cells — independence from estrogen receptors and multidrug resistance gene, *Biochem. Biophys. Res. Commun.*, 179, 661–667, 1991.

- Pettersson, H. and Kiessling, K.H., Liquid chromatography determination of the plant estrogens coumestrol and isoflavones in animal feed, *J. Assoc. Off. Anal. Chem.*, 67, 503–506, 1984.
- Plewa, M.J., Wagner, E.D., Berhow, M.A., Conway, A., Rayburn, A.L., and Anderson, D., Antimutagenic activity of chemical fractions isolated from a commercial soybean processing by-product, *Teratogenesis Carcinogen. Mutagen.*, 19, 121–135, 1999.
- Pratt, D.E. and Birac, P.M., Source of antioxidant activity of soybeans and soy products, *J. Food Sci.*, 44, 1720–1722, 1979.
- Price, K.R. and Fenwick, G.R., Naturally occurring oestrogens in food — a review, *Food Addit. Contam.*, 2, 73–106, 1985.
- Pueppke, J.L., The genetics and biochemical basis for nodulation of legumes by rhizobia, *Crit. Rev. Biotechnol.*, 16, 1–51, 1996.
- Reinli, K. and Block, G., Phytoestrogen content of foods — a compendium of literature values, *Nutr. Cancer*, 26, 123–148, 1996.
- Rivera-Vargas, L.I., Schmitthenner, A.F., and Granham, T.L., Soybean flavonoid effects on metabolism by *Phytophthora sojae*, *Phytochemistry*, 32, 851–957, 1993.
- Rong, H., Stevens, J.F., Deinzer, M.L., De Cooman, L., and Keukeleire, D., Identification of isoflavones in the roots of *Pueraria lobata*, *Planta Med.*, 64, 620–627, 1998.
- Sachse, J., Quantative hochdruckflüssigchromatographie von isoflavonen in rotklee (*Trifolium pratense* L.), *J. Chromatogr.*, 298, 175–182, 1984.
- Seo, A. and Morr, C.V., Improved high-performance liquid chromatographic analysis of phenolic acids and isoflavonoids from soybean protein products, *J. Agric. Food Chem.*, 32, 530, 1984.
- Setchell, K.D.R. and Adlercreutz, H., Mammalian lignans and phytoestrogens: recent studies on their formation, metabolism and biological role in health and disease, in *Role of the Gut Flora in Toxicity and Cancer*, Rowland, I.R., Ed., Academic Press, San Diego, 1988, pp. 315–345.
- Setchell, K.D.R. and Cassidy, A., Dietary isoflavones: biological effects and relevance to human health, *J. Nutr.*, 129, 758S–767S, 1999.
- Setchell, K.D.R., Lawson, A.M., McLaughlin, L.M., Patel, S., Kirk, D.H., and Axelson, M., Measurement of enterolactone and enterodiol, the first mammalian lignans, using stable isotope dilution and gas-chromatography-mass spectrometry, *Biomed. Mass Spectrom.*, 10, 227–235, 1983.
- Setchell, K.D.R., Borriello, S.P., Hulme, P., Kirk, D.N., and Axelson, M., Nonsteroidal estrogens of dietary origin: possible roles in hormone-dependent disease, *Am. J. Clin. Nutr.*, 40, 569–569, 1984.
- Setchell, K.D.R., Welsh, M.B., and Lim, C.K., High-performance liquid chromatographic analysis of phytoestrogens in soy protein preparations with ultra-violet, electrochemical and thermospray mass spectrometric detection, *J. Chromatogr.*, 386, 315–323, 1987.
- Setchell, K.D.R., Childress, C., Zimmer-Nechemias, L., and Cai, J., Method for measurement of dietary secoisolariciresinol using HPLC with multichannel electrochemical detection, *J. Med. Food*, 2, 193–198, 1999.
- Sharma, R.D., Isoflavonoids and hypercholesterolemia in rats, *Lipids*, 14, 535–540, 1979.
- Shihabi, Z.K., Kute, T., Garcia, L.L., and Hindsdale, M., Analysis of isoflavones by capillary electrophoresis, *J. Chromatogr.*, 680, 181–185, 1994.
- Shiizaki, K., Goto, K., Ishige, A., and Komatsu, Y., Bioassay of phytoestrogen in herbal medicine used for postmenopausal disorder using transformed MCF-7 cells, *Phytother. Res.*, 13, 498–503, 1999.

- Shutt, D.A. and Cox, R.I., Steroid and phytoestrogen binding to sheep uterine receptors *in vivo*, *J. Endocrinol.*, 52, 299–310, 1972.
- Song, T., Barua, K., Buseman, G., and Murphy, P.A., Soy isoflavone analysis: quality control and a new internal standard, *Am. J. Clin. Nutr.*, 68(Suppl.), 1473S–1479S, 1998.
- Thompson, L.U., Robb, P., Serraino, M., and Cheng, F., Mammalian lignan production from various foods, *Nutr. Cancer*, 16, 43–52, 1991.
- Van de Castele, K., Geiger, H., and Van Sumere, C.F., Separation of flavonoids by reversed-phase high-performance liquid chromatography, *J. Chromatogr.*, 240, 81–94, 1982.
- Vänttinen, K. and Moravcova, J., Phytoestrogens in soy foods: determination of daidzein and genistein by capillary electrophoresis, *Czech. J. Food Sci.*, 17, 61–67, 1999.
- Wang, C., Sherrard, M., Pagadala, S., Wixon, R., and Scott, R.A., Isoflavone content among maturity group 0 to II soybeans, *J. Am. Oil Chem. Soc.*, 77, 483–487, 2000a.
- Wang, G., Kuan, S.S., Francis, O.J., Ware, G.M., and Carman, A.S., A simplified HPLC method for the determination of phytoestrogens in soybean and its processed products, *J. Agric. Food Chem.*, 38, 185–190, 1990.
- Wang, G.J., Lapčík, O., Hampl, R., Uehara, M., Al-Maharik, N., Stumpf, K., Mikola, H., Wähälä, K., and Adlercreutz, H., Time-resolved fluorimmunoassay of plasma daidzein and genistein, *Steroids*, 65, 339–348, 2000b.
- Wang, H.J. and Murphy, P.A., Isoflavone content in commercial soybean foods, *J. Agric. Food Chem.*, 42, 1666–1673, 1994.
- Wang, J. and Sporns, P., MALDI-TOF MS analysis of isoflavones in soy products, *J. Agric. Food Chem.*, 48, 5887–5892, 2000.
- Wang, W., Radioimmunoassay determination of formononetin in murine plasma and mammary glandular tissue, *PSEMB*, 217, 281–287, 1998.
- Wang, W.G., Tanaka, Y., Han, Z.K., and Cheng, J., Radioimmunoassay for quantitative analysis of formononetin in blood plasma and rumen fluid of wethers fed red clover, *J. Agric. Food Chem.*, 42, 1584–1587, 1994.
- Weinberg, D.S., Manier, M.L., Richardson, M.D., and Haibach, F.G., Identification and quantification of anticarcinogens in garlic extract and licorice *Glycyrrhiza* sp. root extract powder, *J. High Res. Chromatogr.*, 15, 641–654, 1992.
- West, L.G., Birac, P.M., and Pratt, D.E., Separation of the isomeric isoflavones from soybeans by high-performance liquid chromatography, *J. Chromatogr.*, 150, 266, 1978.
- Westcott, N.D. and Muir, A.D., Overview of flax lignans, *Intern. News Fats Oils Rel. Mat.*, 11, 118–121, 2000.
- Westcott, N.D., Muir, A.D., and Northrup, S.F., Analysis of flax lignans after enzymatic and chemical extraction, Proc 57th Flax Institute U.S., Fargo, ND, 1998, 27–30.
- Wilcox, G., Wahlquist, M.L., Burger, H.G., and Medley, G., Oestrogenic effects of plant foods in postmenopausal women, *Br. Med. J.*, 301, 905–906, 1990.
- Wilson, T.C., Harris, R.K., Winters, T.R., Greaves, J.G., and Haggerty, W.T., Supercritical fluid extraction of polar materials from animal feeds and plant matrices, 205th ACS National Meeting, Denver, CO, *Am. Chem. Soc.*, 33, 391–394, 1993.
- Zeleniuch-Jacquotte, A., Adlercreutz, H., Akhmedkhanov, A., and Tonido, P., Reliability of serum measurements of lignans and isoflavonoid phytoestrogens over a two-year period, *Cancer Epidemiol. Biomarkers Prev.*, 7, 885–889, 1998.

2 Analysis of Fatty Acids in Functional Foods with Emphasis on ω 3 Fatty Acids and Conjugated Linoleic Acid

Gary Dobson

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2.1 ROLE OF FATTY ACIDS IN HEALTH

Fatty acids are ubiquitous molecules in biological systems. They occur as components of lipids, notably, phospholipids and glycolipids in membranes and triacylglycerols in seed oils of plants, oily fish, and adipose tissue (fat) in animals. They are present in appreciable quantities in many foodstuffs, and although there has been a great deal of concern over the consumption of too much fat, it has to be remembered that fatty acids are essential to our well-being.

There is a range of different types of fatty acids, varying in chain length and number of double bonds (degree of unsaturation), and any one source normally contains several different types. Fatty acids with even numbers of carbon atoms

predominate and there are short chain (C_4 to C_{10}), medium chain (C_{12} , C_{14}), long chain (C_{16} to C_{22}), and very long chain ($>C_{22}$) fatty acids; sometimes the C_{20} to C_{24} fatty acids are referred to as long chain. Common saturated fatty acids are palmitic (16:0) and stearic (18:0) acids. Important unsaturated fatty acids in nutrition include the monounsaturated oleic acid (18:1 ω 9; the double bond is 9 carbons from the terminal methyl end of the molecule), diunsaturated linoleic acid (LA; 18:2 ω 6; containing two methylene-interrupted double bonds, the one nearest the terminal methyl is 6 carbons down from it), triunsaturated α -linolenic acid (ALA; 18:3 ω 3), tetraunsaturated arachidonic acid (AA; 20:4 ω 6), pentaunsaturated eicosapentaenoic acid (EPA; 20:5 ω 3), and hexaunsaturated docosahexaenoic acid (DHA; 22:6 ω 3). It can be seen from this list that some unsaturated fatty acids are of the ω 3 (or n -3) type, whereas others are ω 6 (or n -6), with the fatty acids in each group being biosynthetically related. LA and ALA are termed essential fatty acids because they cannot be biosynthesized by humans and they must be provided in the diet from vegetable or animal sources. The more unsaturated and longer ω 6 and ω 3 acids (Figure 2.1) may be biosynthesized from LA and ALA, respectively, or they may be obtained from the diet. The double bonds in unsaturated fatty acids can exist in two geometrical forms. The *cis* form overwhelmingly predominates in nature, although *trans* fatty acids occur naturally in ruminant milk and tissues. *Trans* fatty acids are also formed in partially hydrogenated oils used in foods.

The simple health message has been to eat less saturated fat and more unsaturated fat. Saturated fatty acids, in general, increase the risk of cardiovascular disease by raising low-density lipoprotein (LDL) cholesterol. However, not all saturated acids are equal in this respect; lauric (12:0), myristic (14:0), and palmitic acids are more harmful than stearic acid, which appears to be neutral in its effect on LDL cholesterol (Molkentin, 1999). It is generally considered that oleic acid decreases LDL cholesterol levels. It is interesting that lauric acid has been implicated as having anti-cancer properties (Enig, 1994), and infants require medium-chain fatty acids (MCFA) as a rapid energy source (Willis, Lencki, and Marangoni, 1998), thus highlighting the oversimplification of whether a fatty acid is either "good" or "bad." Butyric acid (4:0), present in bovine milk, is also thought to have anticarcinogenic properties, and short-chain fatty acids, in general, have been shown to reduce serum cholesterol and triglyceride levels (Molkentin, 1999). The role of *trans* fatty acids in nutrition is controversial, but there is considerable evidence that they raise triglycerides and LDL cholesterol and lower high-density lipoprotein (HDL) cholesterol, i.e., they may increase the risk of cardiovascular disease.

Over the past few years, interest in the role of fatty acids in health has focused on long-chain polyunsaturated fatty acids (PUFA), particularly AA, EPA, and DHA. This topic has been covered by several reviews (Horrocks and Yeo, 1999; Schmidl, 1996; Simopoulos, 1994; Uauy-Dagach and Valenzuela, 1996), and only the main points will be outlined here. First, DHA and AA, present in human milk, are essential for normal visual and cerebral function in infants. Second, the ω 3 fatty acids, DHA and EPA, cause a number of effects that are considered to protect against cardiovascular disease. These effects include lowering of triglyceride levels by decreasing very low-density lipoprotein (VLDL) synthesis, antithrombotic activity by decreasing platelet aggregation, lowering of blood pressure, and antiatherogenic activity.

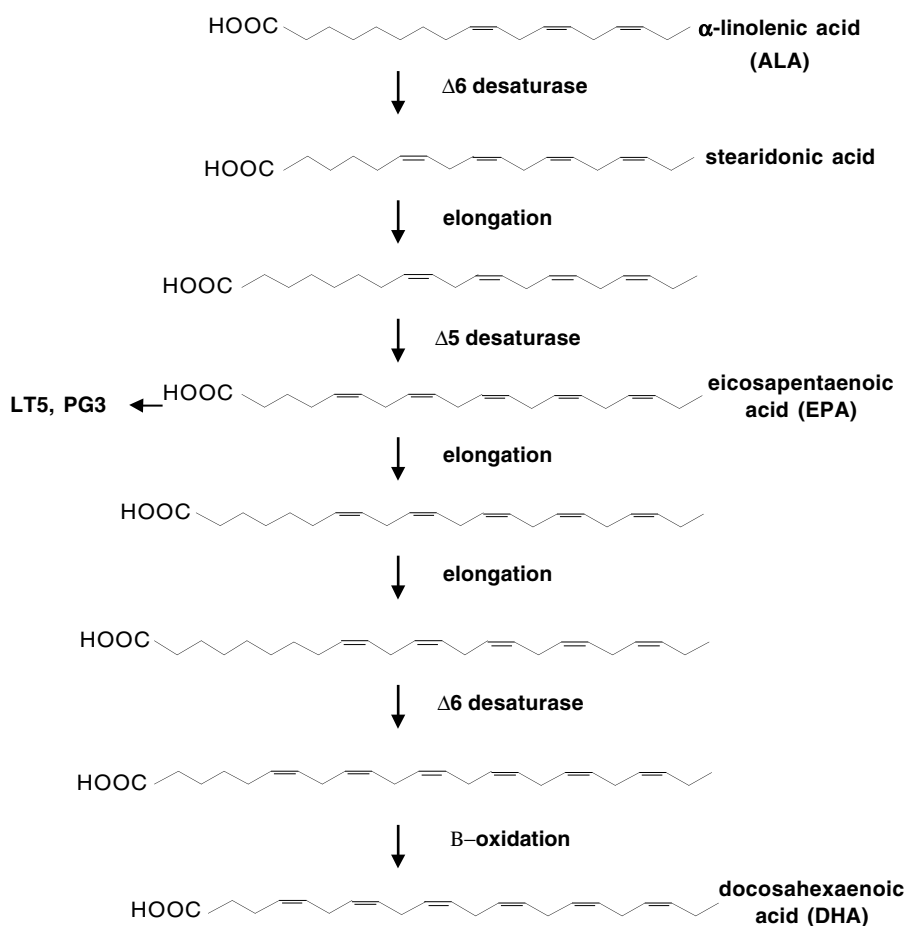


FIGURE 2.1 Biosynthetic pathway of ω 3 fatty acids. Abbreviations: LT5, series-5 leukotrienes; PG3, series-3 prostaglandins.

Antiinflammatory effects (involved in arthritis, psoriasis, and ulcerative colitis, for example) and involvement in restricting tumor growth and in reducing autoimmune response have also been implicated. EPA and AA (and dihomo- γ -linolenic acid), present in membrane phospholipids, are precursors of eicosanoids (comprising prostaglandins, leukotrienes, and thromboxanes) that exhibit a variety of physiological functions such as regulating the inflammatory response and platelet aggregation. The structures of the eicosanoids from the two fatty acids are different, and they have separate and competitive functions; those from AA are more prothrombotic and proinflammatory. The balance of the different types of eicosanoids, determined by the ratio of AA and EPA, influences the physiological outcome, with those from EPA resulting in a more favorable response (antithrombotic, antiinflammatory, and antivasoconstrictive). It is now recognized that the ratio of ω 6 to ω 3 in the modern diet is generally too high (20 to 30:1) for optimal health, and therefore, it is

recommended to consume more $\omega 3$ fatty acids (present in high concentrations in oily fish) and less $\omega 6$ to redress this balance to a ratio of 2 to 4:1.

The role of ALA in increasing levels of EPA and DHA has also received attention. Competition between ALA and LA for the same enzymes that are involved in both the $\omega 3$ and $\omega 6$ pathways influences the level of EPA and DHA. Earlier studies suggested that conversion of ALA to EPA and DHA was low due to the rate-limiting effect of the $\Delta 6$ -desaturase, but there is now evidence that dietary ALA can increase EPA, but less so DHA, in tissue and can affect eicosanoid production. However, the effects of supplementation with ALA are not the same as with EPA and DHA supplementation. Although ALA can reduce blood pressure, it does not decrease plasma triglycerides. It would appear that the role of ALA in health is more tentative than that of EPA and DHA.

Weighing up the beneficial/harmful effects of the various fatty acids, it has been suggested that the ideal dietary fats for adults should be low in cholesterol and *trans* fatty acids; be high in oleic acid, EPA, and DHA; be low in myristic acid; have an LA to ALA ratio of 4:1; and obtain <2% energy from LA (Willis, Lencki, and Marangoni, 1998). Human breast milk is optimum for infants in containing low amounts of *trans*/acids, high AA and DHA but not EPA, a LA to ALA ratio of 7:1, and 4 to 8% MCFA. Palmitic acid is mainly in the 2-position of triacylglycerol for optimum absorption.

Another nutritionally important fatty acid is γ -linolenic acid (GLA; 18:3 $\omega 6$). This $\omega 6$ acid is converted to dihomog-LA (20:3 $\omega 6$), resulting in increased prostaglandin production and decreased inflammation. It has been used to treat rheumatoid arthritis, atopic eczema, and has anti-cancer activity (McDonald and Fitzpatrick, 1998). Stearidonic acid (18:4 $\omega 3$) is a precursor to EPA, and an increase in its consumption may be an efficient way to increase the longer chain $\omega 3$ acids by bypassing the possible rate-limiting step involving the $\Delta 6$ -desaturase conversion of ALA to stearidonic acid.

Conjugated linoleic acid (CLA) is another fatty acid that is currently receiving considerable attention because of a range of properties that may make a positive contribution to health (Banni and Martin, 1998; Cook and Pariza, 1998; Doyle, 1998; Fritsche and Steinhart, 1998; Fritsche et al., 1999; O'Shea et al., 1998; Pariza, 1999; Pariza, Park, and Cook, 2000). CLA is the collective name for a range of conjugated octadecadienoic geometrical and positional isomers. Positional isomers may range from the 6,8 to 13,15 positions, and *cis,trans*, *trans,cis*, *cis,cis*, and *trans,trans* geometrical isomers may occur. CLA can occur naturally at low levels in a range of products, but is highest (about 0.5% of total fat) in ruminants, both in meat and in dairy products. It is also produced on an industrial scale by alkaline isomerization of LA (from sunflower or safflower oil) and is often referred to as commercial CLA. In natural CLA, *cis*-9, *trans*-11 octadecadienoate (9*c*,11*t*-18:2; Figure 2.2) is always the major isomer, formed during microbial biohydrogenation of LA, although a variety of other isomers can occur as minor components. In industrial preparations, the 9*c*,11*t* and 10*t*,12*c* isomers are the major components, but there are varying amounts of the 8*t*,10*c* and 11*c*,13*t* isomers and sometimes other positional isomers. *Cis,cis* and *trans,trans* isomers are also produced, usually as minor components.

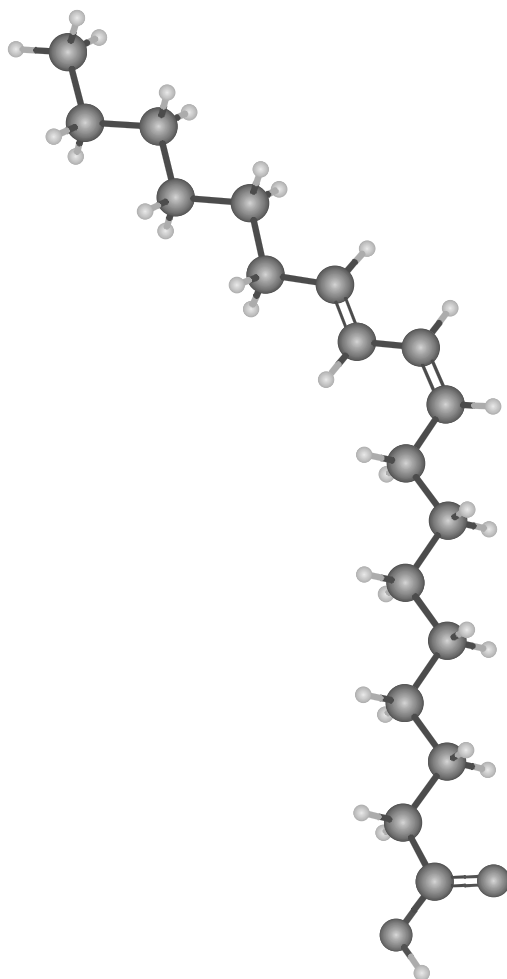


FIGURE 2.2 Structure of *cis*-9, *trans*-11 octadecadienoate (9c,11t-18:2), the major natural CLA.

The physiological properties of CLA have been extensively reviewed (Banni and Martin, 1998; Cook and Pariza, 1998; Doyle, 1998; Fritsche and Steinhart, 1998; O'Shea et al., 1998; Pariza, 1999; Pariza, Park, and Cook, 2000) and include inhibition of carcinogenesis and atherosclerosis, enhancement of immunological function, and effects on body composition by reducing body fat while enhancing lean body mass. Earlier studies on the biological activity of CLA used feeding mixtures that were not well defined in their composition, but probably contained approximately equal amounts of the 9c,11t and 10t,12c isomers. It is now evident that the two types of isomers have different effects. 10t,12c-18:2 has been shown to be the active isomer in relation to changes in body mass composition and to some effects on atherosclerosis and the immune system. The role of the 9c,11t isomer is

less clear. Both isomers seem to be effective in inhibiting carcinogenesis, and the 9*c*,11*t* isomer may have a role in growth enhancement of young rodents. There appear to be different mechanisms by which the two isomers exert their effect, but alterations in eicosanoid signaling seem to play a major role. It is not known whether other isomers have biological activity. It is worth noting that the active CLA isomers are *trans* fatty acids and this may pose some problems, for example, in labeling of foods and in deciding whether these potentially beneficial acids should be included with the other *trans* acids.

2.2 FATTY ACIDS IN FUNCTIONAL FOODS

The obvious way to increase $\omega 3$ intake is to eat more fish, but many people do not like the taste of fish. Taking a fish oil supplement such as cod liver oil is another possibility, but a more attractive proposition is to be able to obtain $\omega 3$ acids from a variety of foods that do not normally contain these acids. Therefore, a number of functional foods to which $\omega 3$ acids have been added have been developed. Such foods are more established in Japan than in Europe and the United States (Garcia, 1998). It is interesting that in Japan, where $\omega 3$ -enhanced foods range from bread to meat products (e.g., sausages) and soft drinks, the emphasis is often on the benefits of DHA for the brain rather than for the cardiovascular system (Hilliam, 1996). The $\omega 3$ acids can be added in a variety of different forms. The simplest form is fish oil with a typical $\omega 3$ content of about 15 to 25% and a variable ratio of EPA to DHA depending on the origin. For example, EPA predominates over DHA in herring and menhaden, but the reverse is true for salmon and tuna (Padley, Gunstone, and Harwood, 1994). Therefore, there is a range of oils that can be used for different applications. For example, low EPA oils are used for infant formulae because EPA can produce negative effects such as reducing body weight (Willis, Lencki, and Marangoni, 1998). Oils containing about 40% DHA but no EPA, developed from the alga *Cryptocodinium cohnii*, are particularly useful for this purpose (Becker and Kyle, 1998; Haumann, 1998). $\omega 3$ concentrates, prepared by winterization or fractional distillation, contain about 30% $\omega 3$ acids, but, if the triacylglycerols are converted to free fatty acids or alkyl esters, higher concentrations can be achieved by using urea fractionation (Shahidi, 1998).

Incorporating the fish oil (essentially all triglycerides) directly into the food is a possibility, but highly unsaturated fatty acids are prone to oxidation, resulting in off-flavors, short shelf-life, and reduction in $\omega 3$ content. In one study (Kolanowski, Swiderski, and Berger, 1999), in which a variety of foods were enriched with fish oils, up to 0.5% EPA/DHA could be incorporated into fat spreads and other products of high sweetness and flavor without detection of fish off-flavors, but even low levels (0.05% EPA/DHA) could not be tolerated in more bland products such as milk and orange juice. Some products such as salad dressing had long (4 months) shelf-lives, whereas in others (e.g., low pH juices) the fish oil was unstable. However, highly refined fish oils have been successfully incorporated into products such as low-fat spreads (containing about 1 to 2% EPA/DHA) and bread (0.2 to 0.5% EPA/DHA) (Gorski, 1997; Madsen, 1998; Newton and Snyder, 1997). In spreads, antioxidants such as vitamin E are added, helping to stabilize the $\omega 3$ acids (Hilliam, 1996).

Microencapsulated marine oils were developed to overcome the problems of oxidation (Shahidi, 1998; Andersen, 1995; Lauritzen, 1994). These are powders (typically containing 10% $\omega 3$ acids) in which the oil and antioxidants are entrapped within a suitable coating, such as gelatine or sucrose, and starch is added to prevent clumping. Microencapsulated powders are used mainly in dry goods such as bakery products and milk powders (Muggli, 1997). They are dispersible in water and are reported to be stable and have a neutral taste. They have been incorporated into a host of products including low-fat spreads, bread, biscuits, fruit bars, diet powder, low-fat cakes, fruit juice, salad dressings, milk drinks, soups, baby follow-on food, and infant formula (Lauritzen, 1994; Muggli, 1997). Infant formula can have a shelf-life of up to 2 years in this form (Andersen, 1995).

ALA in flaxseed has been incorporated into breads, baked goods, cereals, and energy bars, with up to 1 g ALA per bar or cereal serving (Haumann, 1998; Oomah and Mazza, 1998). Although ALA is less susceptible to oxidation than EPA and DHA, it is still a potential problem in flaxseed oil in which the ALA content is high. Microencapsulation of flaxseed oil has been developed to expand the range of potential food applications (Oomah and Mazza, 1998). CLA, also, has been protected from oxidation, experimentally, by encapsulation (Ha et al., 1999).

Eggs fortified with DHA and/or ALA have been developed by feeding chickens with either fish oils, flaxseed oil, or microalgae rich in oil (Haumann, 1998; Sim, 1998; Van Elswyk, 1997). It is interesting that EPA incorporation was always negligible, even with fish oils. Off-flavors from fish oils have been reported to be eliminated by incorporating tocopherols into the feed (Sim, 1998) and are apparently absent from feed containing microalgae (Haumann, 1998). By feeding chickens a mixture of microalgae and flaxseed, 175 mg of DHA and 350 mg total $\omega 3$ acids can be incorporated into each egg and up to 700 mg $\omega 3$ acids (mainly ALA) can be incorporated by feeding flaxseed alone (Haumann, 1998). DHA-rich egg lecithin has also been used in infant formula with the possibility of increased DHA incorporation as phospholipids rather than triglycerides (Haumann, 1998). There have also been trials on feeding flaxseed to chickens, pigs, and ducks and microalgae to farmed salmon and dairy cows to increase the $\omega 3$ acid content (Haumann, 1998).

CLA, mainly as the free fatty acid, is available as a supplement in capsule form. It is the most promising fatty acid for future functional food applications, although it has made little impact as yet. There has been a report of enriching eggs with CLA by adding it to feed (Devitt, Latour, and Watkins, 1998). Apart from adding commercial CLA to foods, there are efforts to enhance the natural CLA levels in dairy milk and meat (O'Shea et al., 1998). Levels of up to 30 mg g⁻¹ of milk fat can be attained, compared to typical values of 3 to 12 mg g⁻¹ (Fritsche and Steinhart, 1998). Many factors, including diet (grass quantity and lushness, rapeseed and soybean oil supplements), breed, age of animal, and processing conditions (e.g., heating and starter cultures), affect the CLA content (Fritsche and Steinhart, 1998; Jahreis, Fritsche, and Kratt, 1999). Supplementation of feed with plant oils high in LA and ALA was particularly successful in enhancing CLA levels. In cheese, aging, processing, type of package, origin of milk, and lipid and protein content all affected the CLA content (Lavillonniere et al., 1998; Lin et al., 1998; Werner, Luedecke, and Schultz, 1992). Ideally, CLA should be enhanced without increasing the *trans*

monoene content, although this may prove to be difficult (Jahreis, Fritsche, and Kraft, 1999; Molkentin, 1999).

Sometimes, in developing a functional food, the aim is to alter more than one aspect of the fatty acid profile. For example, in developing the spread “Pact™” (MD Foods Viby, Denmark), the aim was to encompass an overall “healthy” fatty acid profile (Madsen, 1998). As well as increasing the ω 3 content, the total fat content was reduced, total saturated acids and ω 6 acids were lowered, and *trans* fatty acids were virtually excluded (Madsen, 1998). Another example is “Appetize™” (Bunge Foods USA, Bradley, IL), a shortening from blends of vegetable oils and animal fats, formulated to minimize the disadvantages of animal fats and *trans* fatty acids (present in partially hydrogenated vegetable oils) in raising plasma and LDL cholesterol levels (McDonald and Fitzpatrick, 1998). Cholesterol was removed from the animal fats, *trans* fatty acids were avoided, and the balance of LA and myristic acid was optimized. Indeed, in validating the potential health benefits of a fat, the overall fatty acid composition must be examined, not single components in isolation, because some fatty acids (e.g., ω 3 acids, CLA) have potentially positive health attributes, whereas others (some saturated acids, *trans* acids) may have a negative impact.

2.3 EXTRACTION AND ANALYSIS OF FATTY ACIDS

A fatty acid analysis of a food may be required for a variety of reasons, which in turn may influence the type of analysis to be performed. Information may be required in-house on the composition of the food/ingredients or for monitoring a process or the final product, or it may be required for regulatory reasons, i.e., for labeling or for registration of a product. Consequently, the type and complexity of the information required will vary and will also depend on the regulations in a particular country. Information may be required only on those fatty acids (e.g., ω 3 acids) of “functional” significance, on the ratio of such acids to other fatty acids (e.g., ω 6/ ω 3), or on all fatty acids. In fact, a single method, usually using gas chromatography (GC), often gives information on all the fatty acids present. A fatty acid profile may be obtained in which all fatty acid components are expressed as a weight percent of the total fatty acids. Such information may be all that is required in analyzing, for example, the composition of a fish oil, composed essentially of triacylglycerols, to be used for inclusion in a food. On the other hand, quantification of each fatty acid in terms of milligrams per gram of food may often be required. Labeling requirements may be for total fat and saturated fat only or, additionally, for total monounsaturated and total polyunsaturated fat. The polyunsaturated content may be broken down further into total ω 6 and ω 3, and the ω 3 may be further broken down into individual fatty acids: DHA, EPA, and ALA. Irrespective of the detail, all these labels would require a fatty acid profile to be obtained, but with increasing detail increasing discriminatory power between individual components would be necessary.

In most respects, the approach taken in analyzing the fatty acids of functional foods is similar to that for conventional foods. The steps are to extract the total lipids or fatty acids, convert the fatty acids to a suitable derivative, and analyze the derivatized fatty acids by a suitable chromatographic technique, usually GC with

flame ionization detection (FID), but also using other techniques including gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC). The choice of extraction method will be influenced by the type of food product and the types of lipids present. The analytical method will depend on the types and complexities of the fatty acid mixtures as well as the nature and degree of information required. In conventional foods, the fatty acids will normally be mainly in an esterified form, as triacylglycerols and phospholipids. In functional foods, the fatty acids may also be in these forms, but it is possible that they may have been incorporated in other, less “natural” forms such as alkyl (e.g., ethyl) esters, free fatty acids, or encapsulated oils. The analyst should bear in mind that the form in which the fatty acids occur may influence the analytical approach.

2.3.1 EXTRACTION

Care should be taken to minimize changes in the fatty acid composition of the food prior to analysis. Lipids are susceptible to enzymic lipolysis, and unsaturated fatty acids are susceptible to oxidation. Therefore, food samples should be kept at low temperatures (freezer at -20°C or lower), preferably under an atmosphere of nitrogen or in an oxygen-impermeable wrap, prior to extraction.

Two basic approaches can be taken during extraction. If only the fatty acid composition of the sample is required, as is our concern in this chapter, then the sample can be hydrolyzed or transesterified directly to recover the total fatty acids. The other approach is to extract the total lipids and then, in a second step, release the fatty acids, often while converting them to a suitable derivative for analysis.

In the United States, the total fat is defined as the total fatty acids expressed as triacylglycerols. Therefore, procedures for releasing fatty acids for total fat determination are applicable to analysis of fatty acids per se. In the Association of Official Analytical Chemists (AOAC) official method (AOAC, 1995), the sample is digested either with hydrochloric acid, to hydrolyze lipids and also carbohydrate and protein, with which lipid material may be associated with, or with ammonia for dairy products, in the presence of an internal standard (triundecanoin). The fatty acids are then extracted with a petroleum ether–diethyl ether solvent mixture. As pointed out by Ackman (1999), the potential drawback of using hydrochloric acid is that not all lipids may be hydrolyzed totally to free fatty acids. Presumably, this does not matter if an appropriate fatty acid derivatization procedure (usually to form methyl esters) for free and esterified fatty acids is subsequently employed (see Section 2.3.2), as long as all lipids are extracted. However, extraction may be incomplete by employing a relatively nonpolar solvent mixture. Additionally, in view of the problems of artifact formation under acidic conditions during methylation of some fatty acids, including CLA (see Section 2.3.2), the method may not be the first choice for some applications.

A more universal hydrolysis method is to treat the sample with alkali. Typically, the sample is refluxed with 1 *M* ethanolic potassium hydroxide for 1 h. Recently, a butanolic alkali method (Gertz and Fiebeg, 2000) has been adopted as a German Standard Method. The fatty acids are released as salts, and following acidification the free fatty acids can be extracted with solvent. Multiple extractions with solvent will ensure complete recovery of fatty acids, especially if shorter chain fatty acids

(C₁₂ and below), that have a greater affinity for aqueous mixtures than longer chain acids, are present. Alkaline hydrolysis may result in the formation of small amounts of ethyl esters, particularly if the ratio of ethanol to water is too high, but again these will be subsequently converted to methyl esters, along with the free fatty acids, if an appropriate derivatization procedure is employed. The ethyl esters, however, will be lost if the sample is extracted prior to acidification to recover nonsaponifiables such as sterols. So this step should be avoided, if possible. It is the experience of our laboratory that, compared to methods involving extraction of total lipids, alkaline hydrolysis can give better recovery of fatty acids, at least in certain types of samples such as baby formula powders, probably because of the strong association of lipid with other components and possibly the presence of lipid in a microencapsulated form. If the form (free or esterified) in which the fatty acids occur in the food is unknown, it may be advisable to hydrolyze the sample so that all fatty acids are in the free form and then use an appropriate derivatization method (see Section 2.3.2), although hydrolysis could also be done after lipid extraction. Considering the reasonably harsh alkaline conditions used to extract fatty acids from foods, it is probably not advisable to treat samples containing CLA in this way as isomerization may occur. In this case, it would be preferable to extract the lipids and then, if free CLA is required, to use a milder alkaline hydrolysis procedure.

The total lipids may be extracted from a sample, perhaps because, in addition to the total fatty acids, the lipid composition is to be analyzed. There are many extraction methods available, and further information may be obtained from reviews (Ackman, 1999; Christie, 1989; Shahidi and Wanasundara, 1997). The reader is advised to seek out further methods in the literature relevant to the type of sample under investigation. Some general guidelines will be given below. Lipids are distinguished from other macronutrients by being soluble in organic solvents and insoluble in water. However, lipids are diverse in structure and cover a wide range of polarities; therefore, a single extraction procedure may not extract all lipids. Neutral lipids, such as triacylglycerols, are bound hydrophobically and can be extracted with non-polar solvents such as hexane or diethyl ether, but most foods contain large amounts of water which reduce the effectiveness of diethyl ether, in particular, which is hygroscopic and becomes saturated with water resulting in incomplete extraction. Polar lipids such as phospholipids, the major lipids of egg yolk, for example, are held by stronger bonds, and more polar solvents are required for extraction.

Chloroform–methanol in a ratio of 2:1 (by volume) as in the procedure of Folch, Lees, and Stanley (1957) is generally recognized as an appropriate solvent mixture for extraction of most lipids. In a comparison of various methods for extracting total lipids from different food products, a chloroform–methanol procedure was found to be the most effective (Hubbard et al., 1977). In practice, the extraction mixture also contains water, depending on the amount in the material to be extracted. The Bligh and Dyer procedure (1959) takes the amount of water in the sample into account.

In the Folch method (Folch, Lees, and Stanley, 1957), the sample is homogenized with chloroform–methanol (2:1, v/v) and the extraction is usually repeated with fresh solvent to ensure complete extraction of lipids. The ratio of chloroform to methanol may have to be altered in the initial extraction, depending on the amount of water in the sample, in order to obtain a monophasic system. After extraction,

non-lipid contaminants (e.g., sugars, amino acids) are removed by adjusting the ratio of chloroform–methanol–water/0.88% potassium chloride to 8:4:3 (by volume) to give a biphasic system. After vigorous shaking and allowing the layers to settle, the upper aqueous layer is discarded and the lower organic layer, containing the lipids, is evaporated to dryness. When other extraction methods are used, the Folch wash is often applied to remove contaminants; the extracting solvent is evaporated off, and the residue is suspended in chloroform–methanol–water to 8:4:3.

Even the Folch (Folch, Lees, and Stanley, 1957) and Bligh and Dyer (1959) methods may not extract all lipids. For example, polar acidic phospholipids may not be totally recovered in the Folch washing procedure, but in the Bligh and Dyer method, where a more polar organic phase is employed, only very polar lipids such as phosphoinositides may be lost. However, incomplete recovery of triacylglycerols may also result. So it is apparent that no single extraction method can recover all lipids. Other extraction procedures include the use of diethyl ether and petroleum ether for the extraction of dairy products (Sehat et al., 1998a), where triacylglycerols are by far the major lipid class, and water-saturated *n*-butanol for the extraction of materials high in starch (Morrison, Tan, and Hargin, 1980). Modern methods for extracting lipids include accelerated extraction using microwave procedures (Carrapiso and Garcia, 2000; Garcia-Ayuso et al., 1999) and supercritical carbon dioxide extraction (Carrapiso and Garcia, 2000).

A rapid one-step extraction/methylation procedure has been developed for examining the fatty acid composition of processed foods (Ulberth and Henninger, 1992). The method involves freeze-drying the sample and transmethylation with methanol–HCl/toluene to release the fatty acids as methyl esters. Although freeze-drying is often avoided because of potential problems with alterations in the lipids, the method gave comparable results to those involving extraction with chloroform–methanol and acid hydrolysis–Soxhlet extraction. It would be worth exploring the suitability of the rapid procedure further for a range of food matrices.

Once a lipid or fatty acid fraction has been obtained, care is needed to minimize oxidation. Samples should be kept at low temperatures (–20°C in the freezer) in the presence of an inert atmosphere (usually by flushing tubes with nitrogen) and dissolved in nonpolar solvents (e.g., isohexane) containing an antioxidant (e.g., butylated hydroxytoluene) at an appropriate concentration (about 0.005%).

2.3.2 DERIVATIZATION OF FATTY ACIDS

Fatty acids are normally analyzed as methyl esters. These volatile derivatives have favorable chromatographic properties and are easily prepared. The subject has been reviewed by Christie (1989), where protocols can be found. Broadly, there are two types of common derivatizing reagents, acidic and alkaline. Acidic reagents, including sulfuric acid, hydrochloric acid, boron trifluoride, and acetyl chloride in methanol, will catalyze the formation of methyl esters from both esterified fatty acids (the form in which fatty acids are normally found in foods and the form in which they remain in, for example, a Folch type extraction) and free fatty acids (normally only present in small amounts, but formed when samples are subjected to hydrolysis treatments). On the other hand, alkaline reagents, including potassium hydroxide/methanol and sodium methoxide, transesterify esterified fatty acids only and do

not react with free fatty acids (or *N*-acyl lipids such as sphingomyelin). Most fatty acids, including long-chain PUFA, can be treated with either reagent with no adverse effects. However, some fatty acids, including CLA (see below), form artifacts to varying degrees, depending on the type of reagent and the reaction conditions, with acidic reagents.

For samples in which it is known that only esterified fatty acids are present, sodium methoxide treatment can be recommended. The sample, in toluene, is heated at 50°C for 10 min with 0.5 *M* sodium methoxide in anhydrous methanol. The reaction is stopped with acetic acid, saturated sodium chloride is added, and the fatty acid methyl esters are extracted twice with isohexane. The isohexane extracts are dried over anhydrous sodium sulfate, and the solvent is evaporated under nitrogen or on a rotary evaporator. Samples containing free fatty acids, or when the form of the fatty acids is unknown, can be methylated with 1% sulfuric acid in dry methanol by leaving overnight at 50°C. Sodium chloride solution (5%) is added, and the fatty acid methyl esters are extracted twice with isohexane. The isohexane extract is washed with sodium hydrogen carbonate solution (2%) to remove traces of acid and then treated as above. Boron trifluoride/methanol (12 to 14% w/v) is a widely used reagent, but it should be noted that the use of old reagent or solutions that are too concentrated can result in production of artifacts and loss of PUFA (Christie, 1989). An American Oil Chemists' Society (AOCS) method (AOAC, 1992) for fish oils uses 0.5 *M* sodium hydroxide to hydrolyze the sample to release free fatty acids that are then reacted with boron trifluoride/methanol.

Methylation procedures for use with CLA have been the subject of extensive investigation and have recently been reviewed (Yurawecz, Kramer, and Ku, 1999). CLA, if totally present in an esterified form, can be converted to methyl esters with alkaline reagents such as sodium methoxide without causing any changes. However, under acidic conditions, the major *cis,trans* isomers may isomerize to *trans,trans* isomers and allylic methoxy artifacts may also be formed (Kramer et al., 1997; Ostrowska et al., 2000; Shantha, Decker, and Hennig, 1993; Werner, Luedecke, and Schultz, 1992; Yamasaki et al., 1999; Yurawecz, Kramer, and Ku, 1999). Acid-catalyzed methylation can also form additional CLA by reacting with allylic hydroxy oleates, oxidation products of oleate, present in foods (Yurawecz et al., 1994). Therefore, considerable effort has gone into optimizing procedures for methylating free CLA with or without CLA in an esterified form. This is important because commercial CLA is mainly in the free acid form, and could possibly be added to foods in this form, and because natural samples may also contain small amounts. The reaction time and temperature, as well as the type of reagent, are critical for minimizing isomerization/artifact formation. It is worth noting that under acidic conditions, esterified acids take longer to form methyl esters than free fatty acids, so the risk of isomerization/artifact formation is greater. It follows that samples containing mixtures of free and esterified CLA are the most difficult to handle.

It has been reported that isomerization/artifact formation was unacceptable with HCl/methanol at 80° to 100°C for 60 min (Kramer et al., 1997; Ostrowska et al., 2000; Yamasaki et al., 1999), and even at room temperature for 30 min (Ostrowska et al., 2000). On the other hand, it has been stated that 60°C for 20 min produces minimal isomerization/artifacts (Park and Pariza, 1998), but although triglycerides

were totally methylated under these conditions, phospholipids (a minor component in most dairy products) were not (Kramer et al., 1997). Boron trifluoride/methanol has been used under a variety of conditions, but isomerization/artifact formation was apparent, certainly at temperatures of 70°C and above after only a few minutes (Kramer et al., 1997; Ostrowska et al., 2000; Shantha, Decker, and Hennig, 1993; Werner, Luedecke, and Schultz, 1992; Yamasaki et al., 1999). No detectable changes were observed at room temperature for 30 min (Werner, Luedecke, and Schultz, 1992) but it was not known whether methylation was complete for esterified CLA. Yamasaki and colleagues (1999) found that, compared to hydrochloric acid or boron trifluoride, sulfuric acid resulted in relatively small changes, and under specific conditions (0.87% sulfuric acid in methanol for 2 h at 100°C, in the presence of either dimethylsulfoxide or dimethylformamide to prevent formation of methoxy artifacts), the CLA composition was unchanged. However, the method was tested only on free CLA, although a 2-h reaction time was allowed because it was considered that all esterified CLA would have reacted after this time.

Tetramethylguanidine (TMG) in methanol has been reported to be the only base-catalyzed reagent that can react with both free fatty acids and esterified acids (Schuhardt and Lopes, 1988). However, in one study (Shantha, Decker, and Hennig, 1993), although isomerization/artifacts were not formed, it was ineffective in converting free fatty acids, presumably because strict anhydrous conditions were essential. In another study (Ostrowska et al., 2000), incomplete derivatization and a loss of *trans,trans* isomers were observed. TMG has also been found to give incomplete methylation of phosphatidylcholine and to form compounds that interfered with the GC analysis of low molecular weight fatty acid methyl esters (FAME) present in bovine milk (Kramer et al., 1997).

Trimethylsilyldiazomethane will form methyl esters of free fatty acids only and has been used in several studies for analysis of commercial CLA (Sehat et al., 1998b; Yurawecz et al., 1998). However, it has been observed that, although isomerization was minimal, several artifacts of unknown origin and identity were produced (Kramer et al., 1997). This phenomenon requires further investigation, but it may well be that the artifacts are not derived from CLA.

For natural samples containing mainly esterified and small amounts of free fatty acids, a combination of methods has been recommended (Kramer et al., 1997). The approach was to use an alkaline method for esterified CLA together with a suitable reagent for free CLA, thus avoiding the longer times under acidic conditions required to methylate esterified CLA. Either sodium methoxide, followed by a mild acid method (hydrochloric acid, 80°C for 10 min, or boron trifluoride, 50°C for 10 min), or diazomethane–sodium methoxide were suggested, although the former still gave minor isomerization of CLA. In the light of the recent findings of Yamasaki and colleagues (1999), a combined sodium methoxide–sulfuric acid method (possibly in the presence of dimethylsulfoxide or dimethylformamide, but with a shorter reaction time than 2 h because only free acid is to be methylated) may be worth investigating, as might a combined trimethylsilyldiazomethane–sodium methoxide method. All these methods could be applied to samples containing esterified CLA with varying amounts of free CLA. Alternatively, it is worth considering carrying out a mild alkaline hydrolysis (e.g., using 0.1 M potassium hydroxide in 90%

aqueous ethanol and leaving overnight at room temperature) of the total lipid sample to convert all the CLA to the free form and then choosing a suitable methylation procedure, e.g., trimethylsilyldiazomethane or mild acid conditions.

Samples such as dairy products that contain short-chain (C_4 to C_{10}) acids need special treatment during derivatization because of the increased volatility and solubility of these acids in aqueous solutions. Derivatized samples should be extracted with the required volume of solvent for subsequent analysis by GC so that volume reduction by evaporation of solvent is avoided to reduce losses of short-chain esters. Solvents, more polar than hexane or isohexane, such as diethyl ether may be used for extraction, and multiple extraction of the aqueous phase (which may contain a high proportion of salt) may be performed to minimize losses. Methods are available in which there are no aqueous extraction or solvent removal steps and in which the reaction is carried out at room temperature (Christie, 1989). Derivatives other than methyl esters, namely, isopropyl esters (Wolff, Bayard, and Fabien, 1995) and butyl esters (Iverson and Sheppard, 1986), have been prepared for such samples. Isopropyl esters have other advantages for quantitation of short-chain acids (see later). These esters can be prepared by using procedures where methanol is replaced by isopropanol or butanol, for example, using sulfuric acid or sodium isopropoxide/butoxide as catalyst. Isopropyl esters have been prepared from cheese fat using isopropanol/sulfuric acid to determine the CLA content (Lavillonniere et al., 1998). However, presumably there are the same potential problems as with acidic methods for making CLA methyl esters.

2.3.3 QUALITATIVE AND QUANTITATIVE ANALYSIS OF FATTY ACIDS

As already indicated, the analyst may not be interested in analyzing only the “functional” fatty acids, but may also require information on the other fatty acids for a variety of reasons. The complexity of the fatty acid mixture will depend not only on the presence of the functional component, for example, fish oil, but also on other fatty acid components of the food. The fatty acid profile of fish oil is reasonably complex because of the range of chain lengths and degrees of unsaturation and the presence of positional isomers. The additional presence of a partially hydrogenated vegetable oil (containing *trans* fatty acids), coconut or palm kernel oil (containing short-chain acids), or butterfat (containing *trans* fatty acids, short-chain acids, and CLA), for instance, would complicate the mixture further. The analyst may be faced with samples in which not of all the component parts are known.

The standard approach is to analyze the FAME by GC, and, fortunately, the majority of fatty acids can be separated using modern capillary columns. Although different types of columns (varying in the type of chemical phase, length, internal diameter, and phase thickness) may be required for different types of mixtures, in practice usually two different columns will suffice, as detailed below.

2.3.3.1 ω 3 and Other Fatty Acids

EPA and DHA are the major ω 3 acids from fish oil, but ALA is a significant minor component and may also be derived from some vegetable oils such as flaxseed,

rapeseed, and soybean oils. Other $\omega 3$ acids that are present in fish oils at low but significant levels include 16:3, 16:4, 18:4, 20:4, 22:5, and possibly 20:3. The $\omega 6$ acids in fish oils are at relatively low abundance. LA is the major $\omega 6$ acid and may be derived from many other sources, including vegetable oils, followed by AA. Other $\omega 6$ acids from fish oil include GLA, 20:2 and 20:3. The $\omega 6$ acids 22:3, 22:4, and 22:5 are negligible in fish oils, but may be present in some foods, for example, in some meat products. Other PUFA, such as 20 and 22 carbon $\omega 9$ acids, may be encountered at low levels. As well as PUFA, a range of saturated and monounsaturated FAME will be present. Palmitic and stearic acids are usually the most abundant saturated acids, and oleic acid is the major monounsaturated acid, but a range of chain lengths and double bond positional isomers ($\omega 11$, $\omega 9$, and $\omega 7$) may be encountered. The reader is referred to Christie (1989) for more information.

Separations of complex FAME mixtures, with chain lengths up to 24 carbons and up to 6 double bonds, including all $\omega 3$ and $\omega 6$ acids, can be separated on capillary columns of medium polarity and length; see Figure 2.3 for a GC profile of FAME from cod liver oil. A polyethylene glycol or Carbowax type column (e.g., Carbowax-20M or CP-Wax 52CB), often of dimensions 25 to 30 m long, 0.25 mm I.D., and a film thickness of 0.2 μm , is preferred because of its stability and the predictable order in which all compounds elute. An analysis on this type of column would normally be the first step for a sample of unknown composition. Typically, in our laboratory the following conditions are used: an initial oven temperature of 170°C for 3 min, followed by an increase to 220°C at a rate of 4°C min⁻¹, and then holding this temperature for 15 min; hydrogen as carrier gas at an initial flow rate of 1 ml/min; a split ratio 50:1. Typically, 1 μl of a sample at a concentration of about 3 mg/ml in isohexane (containing 0.005% butylated hydroxytoluene as an antioxidant) is injected. Shorter chain esters elute before longer chain esters, and, for a given chain length, retention time increases with increasing degree of unsaturation. All FAME of a given chain length elute before all those of chain length two carbons longer; the only exception is that 22:6 elutes between 24:0 and 24:1. Double bond positional isomers are also separated, with the isomer with double bonds nearer the ester group eluting first. For example, 20:4 $\omega 6$ has a shorter retention time than 20:4 $\omega 3$; therefore, the order of elution for C₂₀ esters would be 18:4 $\omega 6$ < 20:0 < 20:1 $\omega 11$ < 20:1 $\omega 9$ < 20:1 $\omega 7$ < 20:2 $\omega 6$ < 20:3 $\omega 6$ < 20:3 $\omega 3$ < 20:4 $\omega 6$ < 20:4 $\omega 3$ < 20:5 $\omega 3$ < 22:0. By using appropriate standards, the identification of peaks can be verified by comparison of retention times. With some exceptions (e.g., *trans* and short-chain acids; see below), a fish oil FAME standard will contain the majority of components likely to be encountered in food samples. Synthetic FAME standards that mimic the composition of fish oils are readily available.

If short-chain acids are present (indicated by a series of peaks eluting just after the solvent front), then GC conditions should be adjusted to include a low-temperature step (often 70°C) before programming up to the normal starting temperature. The use of other esters, particularly isopropyl esters, should also be considered, particularly if the samples contain dairy fats. Details on analysis of fatty acids from dairy fats using isopropyl esters can be found in the publications of Wolff and colleagues (Wolff, 1995; Wolff, Bayard, and Fabien, 1995).

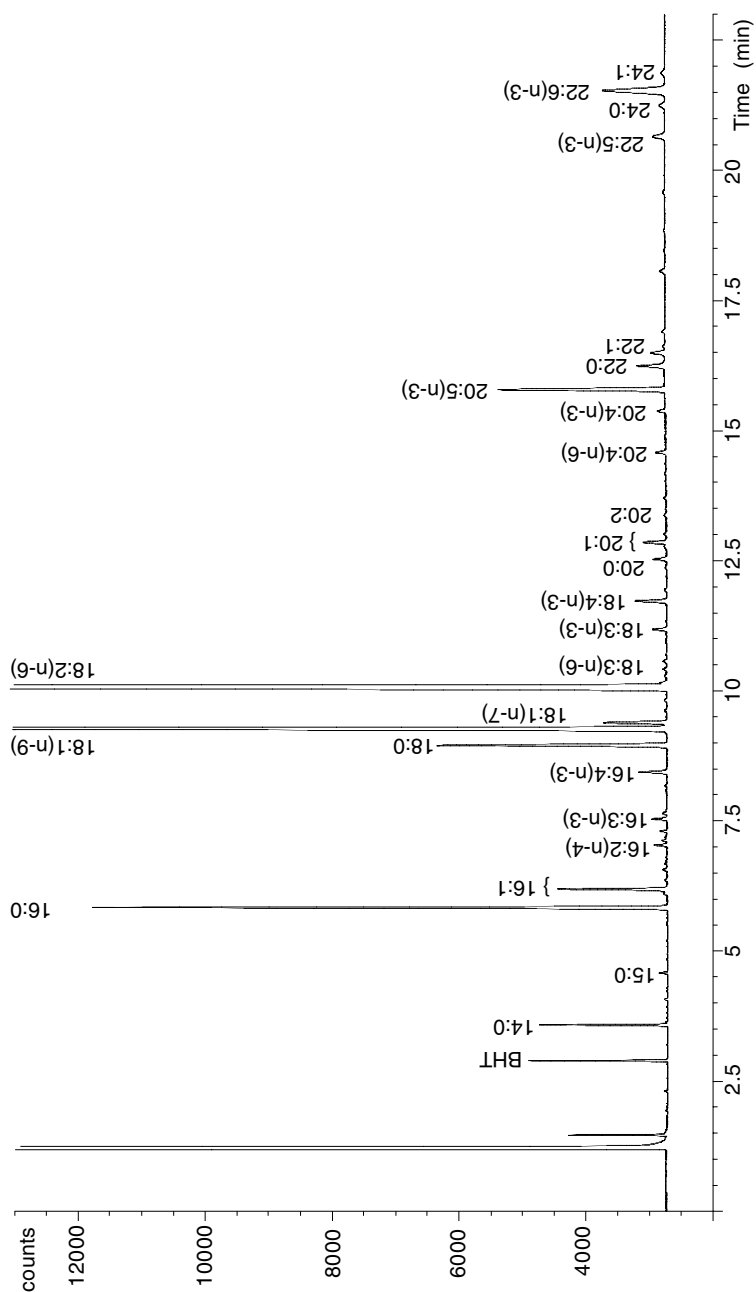


FIGURE 2.3 Partial gas chromatogram of fatty acid methyl esters from a fish/sunflower oil mixture, separated on a CP-Wax 52CB capillary column (25 m $\times$ 0.25 mm I.D. $\times$ 0.2 μ m film thickness; Chrompack BV, Middelburg, The Netherlands). The oven temperature was held at 170°C for 3 min, then was programmed at 4°C min⁻¹ to 220°C, and held at 220°C for 15 min. Hydrogen was the carrier gas at a flow rate of 1 ml min⁻¹, and a split ratio of 50:1 was used. BHT = butylated hydroxytoluene.

From the GC analysis, each FAME can be expressed as a weight percentage of the total FAME. The areas under the GC peaks are approximately equivalent to the masses of the FAME they represent. Therefore, the area percent of each peak (as a percentage of the total areas of all FAME peaks) is approximately equal to the weight percent of each FAME. However, using this approach, the longer chain FAME are overestimated and the shorter chain FAME are underestimated. The area percent values can be multiplied by a theoretical response correction factor (tables are published, e.g., see Christie, 1989), and values are renormalized to give the true weight percent values. The corrections will only make small differences over chain lengths C_{14} to C_{24} , but will make significant differences for shorter chain lengths and may even give inaccurate results for C_4 to C_8 FAME. One of the reasons for analyzing samples containing short-chain acids as isopropyl esters is that, irrespective of chain length, areas are equivalent to masses without the need for correction factors (Wolff, Bayard, and Fabien, 1995). The analyst need only be concerned with short-chain acids and the necessary precautions and method adaptations required to analyze these acids if fatty acids are to be expressed as a percentage of the total fatty acids. When information is required only on the absolute concentration of specific fatty acids (e.g., $\omega 3$ acids), then short-chain acids can be ignored.

To determine the concentration of a fatty acid in terms of milligrams per gram of sample (i.e., the food), a known mass of an internal standard (IS) has to be added to the sample. The IS should be added at the beginning of the extraction procedure (but may be added to a proportion of the extracted fat if the fat content has been determined accurately, gravimetrically), preferably in a form similar to that in which the bulk of fatty acids occurs in the sample (often as triglyceride), although sometimes a FAME IS is used. It must contain a single fatty acid that is either not present in the sample or is at negligible levels, and it should be added at a level so that it represents an average peak size in the GC analysis. Usually, an IS containing an odd chain fatty acid is selected, but the analyst should be aware that odd chain fatty acids are not always absent, and some can be significant components of dairy products. The chain length of the IS should be representative of the fatty acids of a sample; triundecanoin (containing 11:0) may be suitable for a milk fat, but 23:0 IS is more suitable for determining EPA and DHA, as used in AOCS Official Method Ce 1b-89 (1992) for fish oils. It is advisable to carry out the analysis in triplicate and add the IS to two of the samples, but not the third which can serve as a check that the fatty acid component of the IS is not present in the sample (or if it is then it can be allowed for). It is worth noting that even when an IS is added at the beginning of extraction, it will not allow for an inefficient extraction process, because only the lipid in the sample (and not the IS) will be bound in the food matrix. However, the IS may allow for losses in subsequent steps (e.g., washing of organic phase, methylation), certainly if it is structurally similar to the lipids in the sample.

The amount of a FAME in a sample analyzed by GC as FAME can be calculated from the following equation:

$$\text{FAME (mg g}^{-1}\text{)} = \frac{(A_x)(W_{IS})(CF_x)}{(A_{IS})(W_s)} \times 1000$$

where

A_x = area for FAME X

A_{IS} = area for the internal standard

W_{IS} = weight of the internal standard added to the sample (mg)

W_s = weight of the sample (mg)

CF_x = theoretical response correction factor of FAME x relative to that of the IS, e.g., a value of 0.98 for EPA relative to 23:0 as the IS

The previous equation assumes that the IS was added as a FAME. If it was added as a triglyceride, then the equation would also give the concentration of X as triglyceride. Indeed, in the United States, total fat is expressed as the sum of TG (triglyceride) equivalents. However, if the IS was added as a FAME, but the concentration is required in TG equivalents, then the equation would include another correction factor on the denominator. The correction factor would be calculated by dividing three times the molecular weight of the IS as FAME by the molecular weight of the IS as triglyceride. In practice, the correction will be small; e.g., the value is 1.004, with 23:0 methyl ester as the IS.

Performing the calculation for every fatty acid will then allow the amount of any chosen group of fatty acids to be calculated. For example, the total PUFA or total $\omega 3$ can be calculated simply by summing the relevant values for individual FAME. In addition, the ratio of $\omega 6$ to $\omega 3$ can be determined.

Usually, identification by GC, using appropriate standards, is adequate for most purposes. Sometimes GC-MS is used to unambiguously verify the identity of a fatty acid. Low-bleed Carbowax columns (e.g., Supelcowax 10) can be used for this purpose. Methyl esters will normally only give molecular weight information so that the chain length and degree of unsaturation can be verified. In order to determine the exact structure of the fatty acid, including position of the double bonds, other derivatives are used. 4,4-Dimethyloxazoline (DMOX) derivatives are often used for this purpose (Dobson and Christie, 1996). DMOX derivatives have similar chromatographic properties to methyl esters, eluting slightly later, and resolution of peaks is usually retained. In the mass spectra, simple radical-induced cleavage occurs at every carbon in the chain, resulting in gaps of 14 amu between adjacent methylene groups in a saturated chain and a gap of 12 amu when a double bond is encountered (Dobson and Christie, 1996). For example, in the mass spectrum of the DMOX derivative of EPA (Figure 2.4), gaps of 12 amu between m/z 300 and 312, 260 and 272, 220 and 232, and 180 and 192 establish double bonds at $\Delta 8$, 11, 14, and 17. The odd-numbered mass at m/z 153 is characteristic for the remaining double bond at $\Delta 5$.

2.3.3.2 *Trans* Fatty Acids

For many samples, analysis on a Carbowax column may be all that is required. However, columns of this type provide only limited information for some samples, particularly when *trans* fatty acids are present. On Carbowax columns, the presence of *trans*-18:1 methyl esters, from either partially hydrogenated oils or dairy fat, is indicated by the occurrence of peaks after oleate [and after *cis*-vaccenic acid, *cis*-18:1($\omega 7$), often occurring with oleate but at lower levels]. *Trans*-18:1 esters are

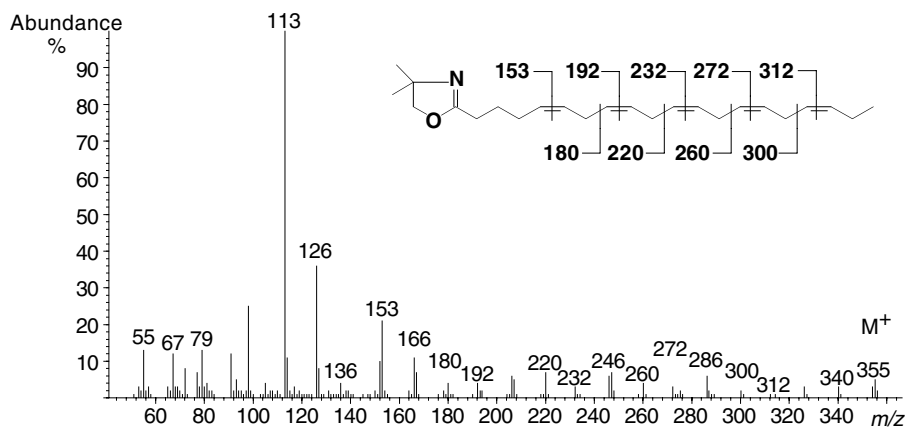


FIGURE 2.4 Electron impact mass spectrum of the 4,4-dimethyloxazoline derivative of EPA.

composed of a number of positional isomers that are poorly separated from a range of *cis*-18:1 isomers (also present in partially hydrogenated fats). Of course, if differentiation between *cis* and *trans* isomers is not required, then the Carbowax column may be adequate. A range of methods has been developed to identify and quantify *trans* isomers. Detailed descriptions of the methods involved are beyond the scope of this chapter (although the absence or low levels of *trans* acids in a food can be considered as a positive nutritional attribute), and they have been covered adequately elsewhere (Ackman, 1999; McDonald and Mossoba, 1996; Ratnayake, 1998; Wolff, Bayard, and Fabien, 1995; Wolff, Precht, and Molkentin, 1998). Only a brief outline will be given here.

Separations on long (at least 50 m, but preferably 100 m), highly polar columns coated with phases containing either 100% cyanopropyl (e.g., CP-Sil 88 or SP-2560) or a high proportion of these groups (e.g., BPX-70) are required. On these columns, under appropriate conditions (often isothermally at 170° to 180°C), many of the *cis*- and *trans*-18:1 isomers are separated into single peaks (esters with double bonds nearest the ester function eluting first), and the majority of *trans* isomers (6*t* to 14*t*) elute before the *cis* isomers. According to Ratnayake (1998) a 100-m SP-2560 is the most efficient column for separation of *cis*- and *trans*-18:1 FAME. 16*t*-18:1 elutes between 13*c*- and 14*c*-18:1 and only 15*t*-18:1, a minor acid in partially hydrogenated fat and dairy fat, overlaps with *cis* isomers. We have observed similar separations with a 100-m CP-Sil 88 column. It should be noted that on cyanopropyl columns, in contrast to Carbowax columns, although retention time increases with chain length and degree of unsaturation, there is considerable chain length overlap.

Silver-ion chromatography can separate FAME according to the number, geometry (*cis* double bonds are held stronger than *trans*), and even positions of the double bonds (Dobson, Christie, and Nikolova-Damyanova, 1995). GC analysis of a *trans* monoene fraction, isolated by either silver-ion thin-layer chromatography (TLC) or HPLC, will provide an accurate assessment of the *trans* monoene isomer distribution. A comparison of the GC profiles of the total FAME with a fraction containing *trans* monoenes and saturated FAME forms the basis of a method considered to be the

most accurate way for quantitatively determining the total *trans* monoene content (Ratnayake, 1998). Silver-ion chromatography is also a useful tool for simplifying any complex fatty acid methyl ester sample into fractions according to number of double bonds (Dobson, Christie, and Nikolova-Damyanova, 1995) prior to GC or GC-MS analysis.

Alternative methods using infrared (IR) and Fourier-transform infrared (FTIR) spectroscopy, based around the absorption of *trans* double bonds at 967 cm^{-1} , are also used for determining total *trans* content, but are relatively inaccurate at low *trans* levels (Ratnayake, 1998). Measurement of *trans* levels using IR will include *trans* polyenes as well as monoenes. *Trans* polyenes are formed in partially hydrogenated oils and are also present in refined oils (nonhydrogenated). They are derived by isomerization of *cis* polyenes such as LA and ALA and potentially from highly unsaturated acids in fish oils, but the products have not been fully characterized. The long polar (cyanopropyl) columns, already mentioned, are used to separate *trans* polyenes (McDonald and Mossoba, 1996; Ratnayake, 1998).

2.3.3.3 Conjugated Linoleic Acid

CLA is another type of fatty acid that cannot be fully evaluated by standard methodology, and substantial effort has gone into developing suitable approaches. A complete qualitative and quantitative determination affords a considerable challenge because of the complexity of geometrical and positional isomers. In functional foods, CLA could potentially come from either natural or commercial sources or possibly a mixture of the two, and the isomer distribution will vary between and within these two sources. There are a variety of different approaches that can be taken, and a complete characterization can usually only be obtained by using a combination of methods. In the following paragraphs, the analytical methodology used for characterizing commercial and natural CLA samples will be considered, followed by some recommendations on how the CLA content of a functional food might be determined.

Initial separations of commercial CLA were achieved by GC. It was recognized that long polar columns, similar to those used for separation of “normal” *trans* isomers, were required to give optimum separations (Eulitz et al., 1999; Kramer et al., 1997, 1999; Yurawecz et al., 1999). A 100-m column coated with 100% cyanopropyl, such as CP-Sil 88 or SP-2560, is normally used. Typical conditions involved holding the temperature at 70°C for 4 min, temperature programming at $13^{\circ}\text{C min}^{-1}$ to 175°C and holding at 175°C for 27 min, then temperature programming at $4^{\circ}\text{C min}^{-1}$ to 215°C and holding at 215°C for 31 min — a total run time of 80 min (Eulitz et al., 1999). These conditions have been used for separating natural CLA isomers, for example, in milk (Figure 2.5) (Kramer et al., 1997). The order of elution of isomers was $9c,11t$ [eluting just after $18:3(n-3)$], followed by other *cis,trans* isomers $11c,13t$ and $10t,12c$, then the *cis,cis* isomers ($8c,10c$, $9c,10c$, $10c,12c$, $11c,13c$, in that order), and finally the *trans,trans* isomers, with the $11t,13t$ isomer eluting first followed by $8t,10t$, $9t,11t$, and $10t,12t$, all of which overlapped (Figure 2.6B). $8t,10c-18:2$ had a slightly longer retention time than $9c,11t-18:2$, but at best occurred as a shoulder on the major peak and could not be quantified separately. These two peaks

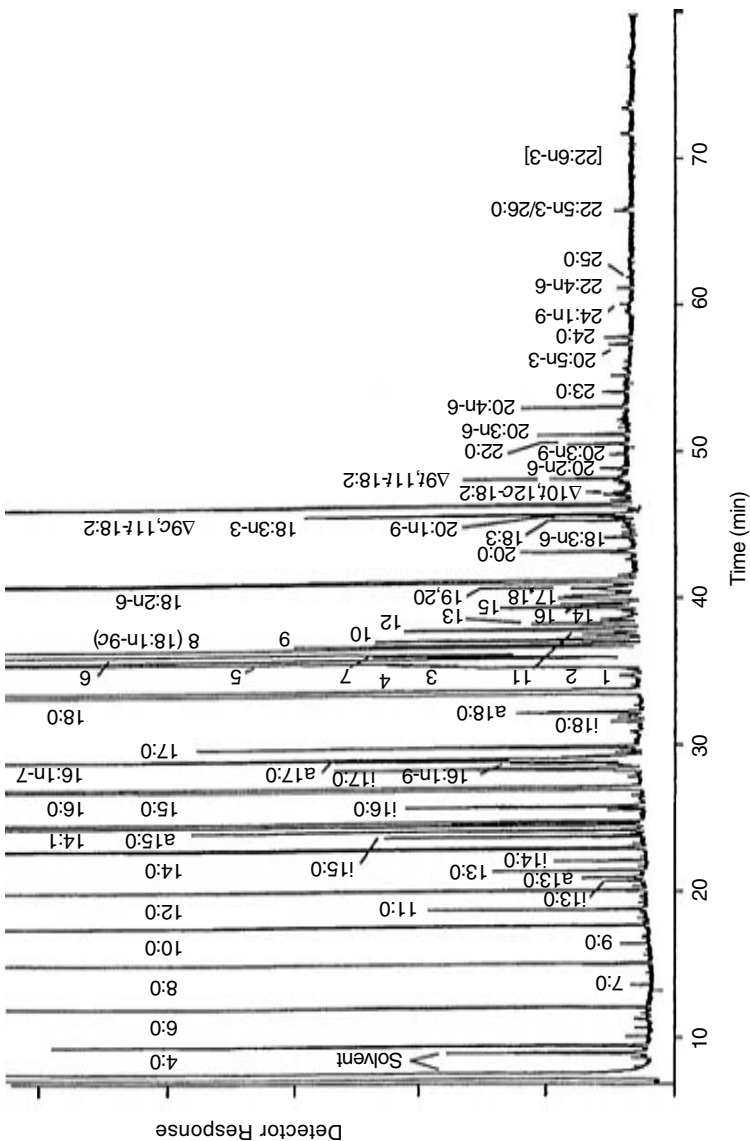


FIGURE 2.5 Gas chromatogram of bovine milk FAME, separated on a SP-2560 capillary column (100 m $\times$ 0.25 mm I.D. $\times$ 0.2 μ m film thickness; Supelco Inc., Bellefonte, PA). The oven temperature was held at 70°C for 4 min, then was programmed at 13°C min⁻¹ to 175°C, was held at 175°C for 27 min, then was programmed at 4°C min⁻¹ to 215°C, and finally held there at 215°C for 31 min. Hydrogen was the carrier gas, and a split ratio of 15:1 was used. Abbreviations: i, iso; a, anteiso; 1 to 20 are cis and trans mono- and diunsaturated FAME. (Reproduced from Kramer, J.K.G., et al., *Lipids*, 32, 1219–1228, 1997. With permission.)

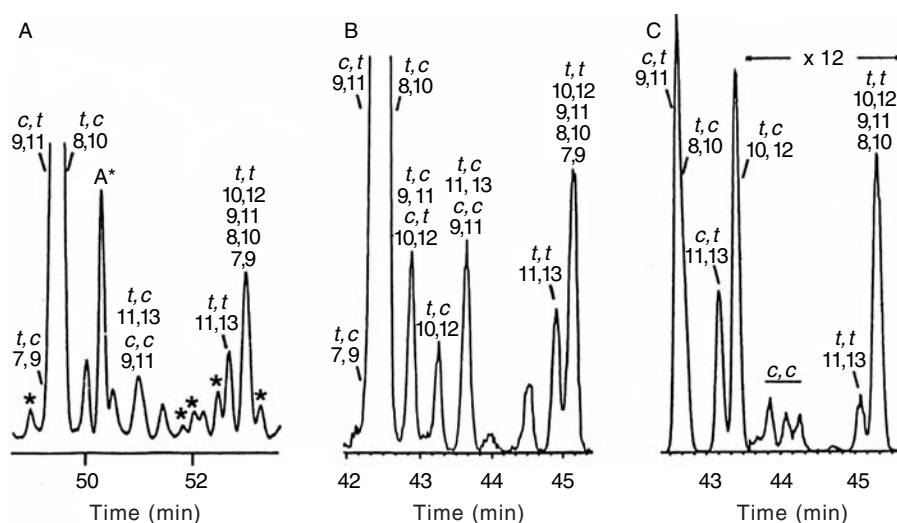


FIGURE 2.6 Comparison of partial gas chromatograms of the conjugated linoleic acid methyl ester (CLA-ME) region from (A) cheese total fatty acid methyl esters with FID, (B) the same region with high-resolution selected ion recording (SIR) at m/z 294.2559 (molecular weight of CLA-ME), and (C) a standard CLA-ME mixture from Nu-Chek-Prep Inc. (Elysian, MN) with high-resolution SIR at m/z 294.2559. Peaks indicated by an asterisk were not found in the SIR chromatogram and are not CLA isomers. Peak A is 21:0. Separations were performed using a CP-Sil 88 capillary column (100 m $\times$ 0.25 mm *i.d.* $\times$ 0.2 μ m film thickness; Chrompack Inc., Raritan, NJ) using hydrogen as the carrier gas. For GC, the oven temperature was held at 75°C for 2 min and then was programmed at 5°C min⁻¹ to 180°C, held at 180°C for 33 min and then was programmed at 4°C min⁻¹ to 225°C, and finally held at 225°C for 43.8 min. For GC-MS, the oven temperature was held at 75°C for 2 min and then was programmed at 5°C min⁻¹ to 170°C, held at 170°C for 40 min and then was programmed at 5°C min⁻¹ to 220°C, and finally held at 220°C for 20 min. (Reproduced from Roach, J.A.G. et al., *Lipids*, 35, 797–802, 2000. With permission.)

could only be resolved on very long (120 m) polar (BPX-70) columns (Christie, Sebedio, and Juaneda, 2001).

Further information on the isomer distribution could be obtained using silver-ion HPLC. The first silver-ion HPLC separation was achieved with a commercial CLA free acid as methyl esters (Sehat et al., 1998b). A ChromSpher 5 (4.6 mm I.D. $\times$ 250 mm) column was employed with 0.1% acetonitrile in hexane as the mobile phase at a flow rate of 1 ml min⁻¹ and UV detection at 233 nm. Isomers were separated into three groups of peaks corresponding to *trans,trans*, *cis,trans*, and *cis,cis* and eluting in that order. Within each group, separation of the 11,13, 10,12, 9,11 and 8,10 positional isomers was achieved to varying degrees, with the isomers always eluting in that order. Compared to GC, the most notable aspects of the separation were the improved separation of the *trans,trans* isomers and the separation of 9*c*,11*t* from 8*t*,10*c*-18:2. Continuous improvement in separation was achieved by increasing the number of silver-ion columns in series up to a maximum of six columns, but three (Eulitz et al., 1999; Sehat et al., 1999) or two (Rickert et al.,

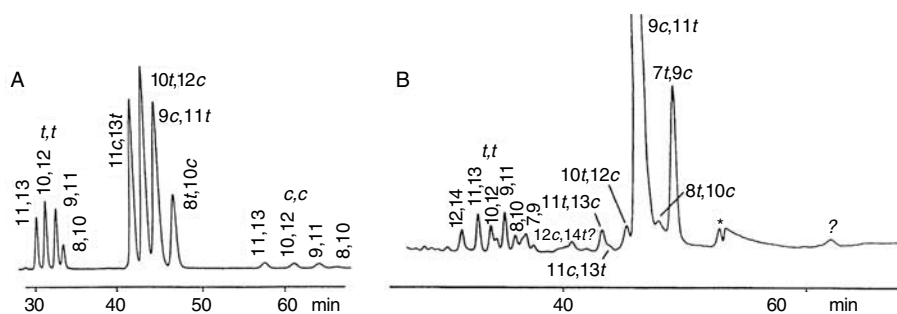


FIGURE 2.7 Silver-ion high-performance chromatogram of the CLA-ME region of (A) a commercial CLA-ME mixture and (B) cheese total fatty acid methyl esters, using three ChromSpher 5 analytical silver-impregnated columns (each 250 mm $\times$ 4.6 mm I.D., 5 μ m particle size; Chrompack, Bridgewater, NJ) and UV detection at either 234 (A) or 233 nm (B). The mobile phase was 0.1% acetonitrile in hexane and operated isocratically at a flow rate of 1 ml min⁻¹. [(A) Reproduced from Eulitz, K., et al., *Lipids*, 34, 873–877, 1999. (B) Reproduced from Sehat, N., et al., *Lipids*, 34, 407–413, 1999. With permission.]

1999) columns were suggested as a good compromise, and all 12 isomers were well resolved (Figure 2.7A). An alternative approach to improving resolution was to use alternative derivatives to methyl esters. Utilizing only a single silver-ion HPLC column, the separation of a commercial CLA mixture was substantially improved as the *p*-methoxyphenacyl esters (Nikolova-Damyanova, Momchilova, and Christie, 2000). Recognizing that methods for forming methyl esters of CLA may cause geometrical isomerization and/or the formation of artifacts, another group (Ostrowska et al., 2000) have recently separated CLA mixtures as free acids by silver-ion HPLC by incorporating acetic acid into the hexane–acetonitrile mobile phase. Separation was slightly improved compared to methyl esters on a single column.

In commercial CLA samples, usually only one of the *cis,trans* forms of each positional isomer (e.g., 9*c*,11*t* but not 9*t*,11*c*) occurs (Eulitz et al., 1999). In natural samples such as cheese, both geometrical isomers may occur (Eulitz et al., 1999; Lavillonniere et al., 1998; Sehat et al., 1998a) and their complete separation is a challenge. To address this problem, Eulitz et al. (1999) prepared all eight *cis,trans* isomers from a commercial CLA sample (containing 8,10, 9,11, 10,12, and 11,13 positional isomers). By silver-ion HPLC using three columns, six peaks were obtained, and all but the 10,12 *cis,trans* pairs were at least partially resolved, although 9*t*,11*c* and 8*c*,10*t* overlapped. GC (100-m CP-Sil 88) separated all but the 9*c*,11*t* and 8*t*,10*c* isomers, although 9*t*,11*c* and 10*c*,12*t* were poorly resolved and the 11*t*,13*c* isomer overlapped with the 9*c*,11*c* isomer. It is interesting that by HPLC the 11*t*,13*c* isomer eluted before the 11*c*,13*t* isomer, but the reverse occurred for the 8,10 and 9,11 isomers, whereas by GC the *cis,trans* isomers always eluted before the corresponding *trans,cis* isomers. In this mixture, all peaks were in roughly similar proportions. In natural samples, some peaks were relatively more abundant than others, resulting in poorer resolution by GC and HPLC.

All the isomers present in commercial CLA have been found in cheese, the food that has been studied in the most detail (Lavillonniere et al., 1998; Rickert et al., 1999; Sehat et al., 1998a, 1999). The 9*c*,11*t* isomer accounted for about 80 to 90% of the total CLA. In addition, application of silver-ion HPLC (Figure 2.7B) (Rickert et al., 1999; Sehat et al., 1998a, 1999) and GC (Figure 2.6A) (Lavillonniere et al., 1998; Sehat et al., 1998a) revealed a range of 7,9 (7*t*,9*c*, 7*c*,9*c*, 7*t*, 9*t*) and 12,14 (12*c*,14*t*, 12*c*,14*c*, 12*t*,14*t*) isomers and 9*t*,11*c*, 10*c*,12*t* and 11*t*,13*c*. 6*t*,8*t* and 13*t*,15*t* isomers were tentatively identified (Rickert et al., 1999). Therefore, with the exception of 7*c*,9*t*, 8*c*,10*t*, and 12*t*,14*c*, all possible geometrical isomers of 7,9 to 12,14 positional isomers have been detected — 21 isomers in total. 7*t*,9*c*-18:2 was often the second major isomer (3 to 16% of the total CLA) and occurred as a shoulder (not quantifiable) by GC at the leading edge of the 9*c*,11*t* peak (Sehat et al., 1998a; Yurawecz et al., 1998), but was resolved as a single component (eluting just after 8*t*, 10*c*) by HPLC only when two (Rickert et al., 1999) or three (Sehat et al., 1999) columns were used.

The *cis,cis* isomers should also be well resolved by silver-ion HPLC, but in natural samples they sometimes appear to be swamped by the oleate peak (Sehat et al., 1999). The GC profile of the CLA-ME region from cheese also contained non-CLA peaks, such as 21:0 and 20:2 isomers, that made identification of CLA isomers difficult without using selected ion recording (SIR; molecular ion at m/z 294) GC-MS (Figure 2.6B) (Eulitz et al., 1999; Roach et al., 2000). 18:4(*n*-3) would also be expected to elute in the CLA region. In another study (Lavillonniere et al., 1998), the total FAME from cheese were subjected to reversed-phase HPLC, and a CLA fraction containing CLA, together with 14:0, 16:1, and LA, was collected prior to analysis by GC. This served to concentrate the CLA isomers and also appeared to eliminate other contaminating peaks in the CLA region of the GC trace.

In determining the identities of the various CLA isomers, a number of other complementary techniques have been employed. Sometimes, CLA was fractionated by semipreparative silver-ion HPLC prior to further analysis (Sehat et al., 1998a,b). GC-MS of the DMOX derivatives readily allowed location of the double bonds (Eulitz et al., 1999; Lavillonniere et al., 1998; Rickert et al., 1999; Sehat et al., 1998a, 1999; Spitzer, Marx, and Pfeilsticker, 1994; Yurawecz et al., 1998). For example, in the mass spectrum of the 8*t*,10*c*-18:2 derivative, gaps of 12 amu between m/z 182 and 194 and m/z 208 and 220 confirmed the position of the double bonds, and similar gaps between m/z 224 and 236 and m/z 250 and 262 confirmed the position of the double bonds in 11*c*,13*t*-18:2 (Figure 2.8) (Sehat et al., 1998a,b). Intense allylic ions at m/z 248 and 290, respectively, were also characteristic. A set of 24 ions was selected to enable identification of all possible CLA isomers in cheese by SIR GC-MS of the DMOX derivatives (Roach, 1999).

GC-MS does not distinguish between geometrical isomers, but this can be achieved by examining the IR spectra obtained by GC-FTIR of the methyl esters (Lavillonniere et al., 1998; Mossoba et al., 1999a; Rickert et al., 1999; Sehat et al., 1998a, 1999; Yurawecz et al., 1998). Two peaks at 978 to 988 cm^{-1} and 946 to 949 cm^{-1} were characteristic of *cis,trans* isomers, but there was only one peak at 986 to 993 cm^{-1} for *trans,trans* isomers, and *cis,cis* isomers lacked peaks in this region of the spectrum. Unsaturated carbon C-H stretch bands at 3002/3020, 3017, and

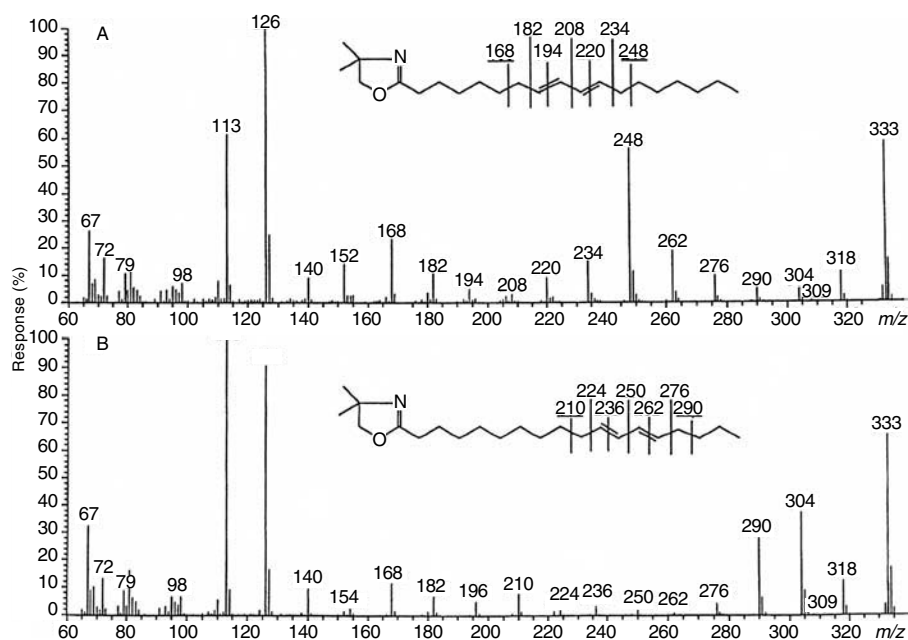


FIGURE 2.8 Gas chromatography-electron impact mass spectra of the 4,4-dimethyloxazoline derivatives of (A) *trans*-8, *cis*-10-18:2 and (B) *cis*-11, *trans*-13-18:2 from a commercial CLA mixture. (Reproduced from Sehat, N., et al., *Lipids*, 33, 217–221, 1998b. With permission.)

3005/3037 cm^{-1} were highly characteristic of *cis,trans*, *trans,trans*, and *cis,cis* isomers, respectively. GC-FTIR does not distinguish between *cis,trans* and *trans,cis* isomers. One way to achieve this was to examine the *cis* and *trans* monoenes, derived from partial hydrazine reduction, by GC-MS of the DMOX derivatives (Lavillonniere et al., 1998).

Information on the isomer distribution in CLA mixtures can be obtained by other approaches. A powerful two-dimensional ^{13}C NMR spectroscopy technique has been used to quantify all 20 isomers (all four geometrical forms of 7,9 to 11,13 isomers) in a complex commercial CLA mixture (Davis, McNeill, and Caswell, 1999a,b). The method was only applied to pure CLA mixtures, and required relatively large sample sizes. Therefore, it would appear that such an approach would be restricted to analyzing commercial CLA mixtures prior to incorporation into foods. Another approach that can be applied to mixtures of CLA with other fatty acids utilized the reactivity of conjugated double-bond systems with the dienophile, 4-methyl-1,2,4-triazoline-3,5-dione, to form Diels-Alder cycloaddition products (Dobson, 1998). The products were analyzed by GC-MS, and the position of the original double bonds could be deduced from the mass spectra. Quantitative information on the distribution of positional isomers could be obtained, but the method did not distinguish between geometrical isomers. The method is useful for complementing other approaches, e.g., in estimating the amount of 8*t*,10*c* isomer that is difficult to separate from 9*c*,11*t*-18:2 by GC.

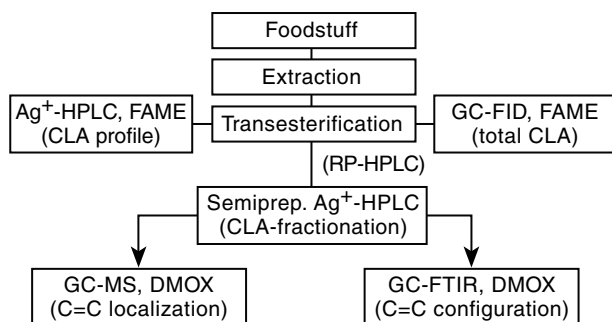


FIGURE 2.9 Proposed flowchart to determine the CLA content and isomer distribution in food. (Reproduced from Fritsche, J., *Lipid Technol. Newsl.*, 4, 115–119, 1998. With permission.)

The previous sections confirm that CLA can be a complex mixture that can be analyzed by a variety of techniques. A complex sequence of analyses has been proposed (Figure 2.9) (Fritsche, 1998; Fritsche et al., 1999), but, although this may be required for a complete unambiguous analysis, it would be time consuming and would require expensive equipment that many laboratories do not have. The degree of sophistication of the adopted analytical approach depends on the type and detail of information required. With the use of appropriate standards, quantitation and tentative identifications of the CLA isomers could be achieved with GC and HPLC alone. The analyst must then decide whether other techniques (e.g., GC-MS of DMOX derivatives, GC-FTIR) are warranted, depending on the required degree of certainty of identifications of the isomers.

In a functional food, at the most basic level the total CLA content may be required. In this case, the total FAME may be analyzed by GC on a long polar column such as a 100-m CP-Sil 88, and the CLA peaks elute between 18:3(ω 3) and 20:2(ω 6) (Figure 2.5) (Kramer et al., 1997; Mossoba et al., 1999b). A commercial CLA mixture would be essential as a standard for identifying the CLA peaks based on retention time. Depending on the source (i.e., commercial or natural) of the CLA, the major CLA isomers will be 9*c*,11*t* (possibly with substantial amounts of overlapping 8*t*,10*c* from some commercial material) with 10*t*,12*c* from commercial CLA and varying amounts of 11*c*,13*t* (a major isomer only in some commercial material). These isomers will account for the majority of CLA, although varying amounts of *trans,trans* isomers occur in commercial CLA. Indeed, sometimes the 9*c*,11*t* isomer (which also includes the 7*t*,9*c* and 8*t*,10*c* isomers) is the only peak that is measured as an estimate of the CLA content in natural samples (Fritsche, 1998; Fritsche and Steinhart, 1998; Fritsche et al., 1999; Lin et al., 1998). Minor CLA peaks may represent a significant minority of the CLA and should also be included. However, some peaks from natural CLA will not be present in the commercial standard, and therefore, it would be advisable to prepare another standard by further isomerization of commercial CLA [using iodine according to Eulitz et al. (1999)] so that all *cis,trans* isomers are present. 7*t*,9*c* would then be the only isomer that may be significant in the sample, but probably not present in the

standard. However, it would be included with the 9*c*,11*t* isomer with which it overlaps by GC. Quantitation, in terms of milligrams of CLA per gram of sample, would be carried out by including an appropriate internal standard during extraction as outlined in Section 2.3.3.1 on ω 3 fatty acids.

A possible problem with using GC alone would be the presence of non-CLA peaks in the CLA region of the chromatogram (Eulitz et al., 1999; Roach et al., 2000; Sehat et al., 1999) that may be mistakenly included as CLA. SIR GC-MS would help to verify CLA peaks (Eulitz et al., 1999; Sehat et al., 1999). Alternatively, prior to GC, a CLA fraction could be isolated by reversed-phase (RP) HPLC, thus minimizing the chance of interfering non-CLA peaks by GC while serving to concentrate minor CLA peaks (Lavillonniere et al., 1998). In this case, quantitation would be achieved by relating the total CLA peak areas to that of LA (isolated in the CLA fraction by RP-HPLC), which in turn would be quantified from the GC trace of the total FAME by reference to an internal standard.

Other approaches to quantitation of total CLA have included supercritical fluid chromatography (SFC) with FID (Yurawecz, Kramer, and Ku, 1999) and RP-HPLC with UV detection at 234 nm (e.g., see Banni et al., 1998). In both methods, CLA isomers eluted as a single or poorly resolved peak. However, the former requires nonroutine equipment, and the latter requires an external calibration procedure. On the other hand, UV detection has the advantage over GC and SFC in that the method has a high degree of specificity (absorption of the conjugated system at 234 nm), thus minimizing problems of misidentification and interference from non-CLA compounds, although there is some interference from isolated double bonds that absorb around 200 to 210 nm (Banni and Martin, 1998). A more accurate RP-HPLC method, where second derivative spectra are used to overcome this problem, is recommended by Banni and Martin (1998). UV spectroscopy of total FAME samples, without chromatographic separation, would not be recommended because of the absorption of other compounds, particularly oxidation products of CLA, also at 234 nm (Banni and Martin, 1998).

The next level of information that may be required could be to quantify (in milligrams per gram of sample) those isomers that are known to be active, i.e., representing the functional CLA. Currently, these are considered to be the 9*c*,11*t* and 10*t*,12*c* isomers, although others may be added as more information is acquired on the biological effects of CLA. This could be achieved by using a combination of GC with FID and silver-ion HPLC with UV detection, again possibly after isolating a CLA fraction by RP-HPLC. GC would be used to quantify single or groups of isomers by reference to an appropriate internal standard. Then, for any one group of geometrical isomers, HPLC would be used to determine the proportion of those isomers that were not resolved by GC. HPLC would not be used to calculate the proportion of isomers between different geometrical groups because they have different extinction coefficients (Hopkins, 1972).

In foods that contain only commercial CLA, the 10*t*,12*c* isomer and the sum of the 9*c*,11*t*/8*t*,10*c* isomers could be quantified using GC (Figure 2.6C) by reference to an appropriate internal standard, and then the ratio of the 9*c*,11*t* and 8*t*,10*c* could be determined by silver-ion HPLC (preferably on two or three columns, Figure 2.7A) with UV detection at 233 nm. Indeed all the components could be quantified by

using a similar approach to that used for a commercial CLA mixture (Sehat et al., 1998b). The amounts of total *cis,trans*, total *cis,cis*, and total *trans,trans* were determined by GC, and then the proportions of the various positional isomers within each group were determined by silver-ion HPLC with UV detection.

The active isomers in natural CLA could also be quantified by using a combination of GC and silver-ion HPLC. The 10*t*,12*c* isomers and the sum of the 9*c*,11*t*/7*t*,9*c*/8*t*,10*c* isomers would be quantified by GC (Figure 2.6A). Then, the proportion of the 7*t*,9*c* and 8*t*,10*c* isomers in the total of 7*t*,9*c*, 8*t*,10*c*, 9*c*,11*t*, 9*t*,11*c*, 10*t*,12*c*, 10*c*,12*t* isomers could be determined by silver-ion HPLC (Figure 2.7B). The same total could be quantified from the GC trace, and hence the 7*t*,9*c* and 8*t*,10*c* isomers would be quantifiable. The amount of 9*c*,11*t* could then be determined by simple subtraction. [Note that the 7*t*,9*c* and 8*t*,10*c* isomers are not determined as a proportion of the total of *all cis,trans* isomers by HPLC, because this total cannot be quantified by GC due to the overlap of 11*t*,13*c* with 9*c*,11*c* (Figure 2.6B)]. By using similar approaches, it should be possible to quantify any isomer in a natural sample.

2.4 CONCLUDING REMARKS

Currently, ω 3 fatty acids (EPA, DHA, and ALA) are the major type of fatty acid incorporated into functional foods because of their potential benefits against cardiovascular disease. The major source of EPA and DHA is fish oils and algae, and it is flaxseed for ALA. ω 3 Fatty acids have also been incorporated into eggs. The analyst who is particularly interested in the ω 3 content may also require a complete qualitative and quantitative analysis of all the fatty acids in the food, perhaps for labeling requirements or for evaluating the total health potential of the food, for this may depend on the balance of various fatty acids which, individually, potentially may have either positive or negative effects on health.

To obtain a total fatty acid profile, to include ω 3 fatty acids, the approach is normally straightforward and involves extraction of lipids or fatty acids in the presence of an appropriate internal standard, derivatization of fatty acids to methyl esters, and analysis by GC. Appropriate extraction methods, depending on the type of food and lipids present, must be employed to extract the total lipids efficiently. An alternative to extracting total lipids is to hydrolyze the sample directly to release all the fatty acids in the free form. An appropriate methylation step is required. Alkaline methods are milder, but will only derivatize esterified fatty acids. Acidic methods are used to derivatize samples containing free fatty acids with or without esterified fatty acids. Separation of FAME can normally be achieved on Carbowax type columns of medium length. Certain precautions or adaptations of protocols may be necessary if certain types of fatty acids, particularly short-chain and *trans* fatty acids, are expected.

There is increasing evidence that CLA may be of potential benefit against cancer and obesity. CLA is currently available in capsule form as a supplement, but there is the potential for incorporation into functional foods in the near future. CLA could come from two main sources: industrially from alkaline isomerization of LA or from dairy products with enhanced levels of CLA (possibly as a result of optimized feeding regimes, processing, etc.). CLA is a complex mixture of positional and

geometrical isomers whose distribution within the two main sources is different. Analysis of CLA requires a considered approach. Special derivatization procedures for forming methyl esters are required because isomerization/artifact formation can occur under acidic conditions. In choosing a procedure, the form (free and/or esterified) of the CLA must be taken into account. The analyst must decide whether the total CLA content is required, the content of the biologically active isomers (9*c*,11*t*- and 10*t*,12*c*-18:2), or a complete assessment of all isomers. The complexity of the analysis increases as more information is required, so that an unambiguous characterization of all isomers involves an array of analytical technologies. A complementary GC and silver-ion HPLC approach and the use of a comprehensive standard, containing most of the isomers, will suffice for most purposes.

Methods involving crystallization (Berdeaux et al., 1998) and lipases (Haas et al., 1999) are available for producing reasonably pure single CLA isomer preparations of both 9*c*,11*t*- and 10*t*,12*c*-18:2. In view of the fact that the two isomers appear to exert different biological effects, it is possible that single isomers may be used to produce different types of functional foods. The two isomers are metabolized in an analogous way to LA into conjugated analogs of long-chain ω 6 PUFA such as AA (Sebedio et al., 1997). These analogs may be more biologically active than CLA itself (Pariza, Park, and Cook, 2000; O'Shea et al., 1998), and, looking further to the future, if they could be prepared on a large scale, they may be incorporated into functional foods. Methods for their analysis are already available (Juaneda and Sebedio, 1999).

Other fatty acid candidates for use in functional foods could include GLA and stearidonic acid. GLA, currently widely available as a supplement, is active against a range of conditions, including rheumatoid arthritis, and is readily available in seed oils of evening primrose, borage, and blackcurrant. Stearidonic acid, also present in blackcurrant oil, is an ω 3 fatty acid from which EPA and DHA are biosynthetically derived. Compared to ALA, stearidonic acid would perhaps be a more effective alternative to EPA and DHA supplementation, because its conversion to the longer chain PUFA is more efficient; the initial Δ 6-desaturation step converting ALA to stearidonic acid is considered to be rate limiting (Figure 2.1). GLA and stearidonic acid are readily analyzed by standard GC methods. Even increasing the content of common fatty acids, notably oleic acid (considered to be beneficial to health), may be considered in functional foods.

Structured lipids in which certain fatty acids are located at specific positions on the glycerol backbone of the triglyceride have been developed mainly as medical foods (Schmidl, 1996; Willis, Lencki, and Marangoni, 1998). For example, triglycerides with MCFA on the 1- and 3-positions and essential and long-chain PUFA in the 2-position have been developed for treatment of fat malabsorption diseases. MCFA are then readily released by pancreatic lipases and absorbed as free fatty acids, acting as a rapid energy source, whereas the PUFA are more readily absorbed as 2-monoglycerides. It is possible that structured lipids may become increasingly important in the functional food market in attempts to optimize the beneficial effects. The implications for the analyst would be that fatty acid positional analyses would be required. A range of methods using specific lipases is available for this purpose.

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REFERENCES

- Ackman, R.G., Application of gas-liquid chromatography to lipid separation and analysis: qualitative and quantitative analysis, in *Fatty Acids in Foods and Their Health Implications*, Chow, C.K., Ed., Marcel Dekker, New York, 1999, pp. 47–65.
- Andersen, S., Microencapsulated omega-3 fatty acids from marine sources, *Lipid Technol.*, 7, 81–85, 1995.
- AOCS Official Method Ce 1b-89, Fatty acid composition by GLC. Marine oils, *American Oil Chemists' Society Official Methods and Recommended Practices*, Firestone, D., Ed., AOCS Press, Champaign, IL, 1992.
- AOAC Official Method 996.06, *Official Methods of Analysis of AOAC International*, 16th ed., AOAC International, Gaithersburg, MD, 1995.
- Banni, S. and Martin, J.-C., Conjugated linoleic acid and metabolites, in *Trans Fatty Acids in Human Nutrition*, Sebedio, J.L. and Christie, W.W., Eds., The Oily Press Ltd., Dundee, Scotland, 1998, pp. 261–302.
- Banni, S., Angioni, E., Contini, M.S., Carta, G., Casu, V., Iengo, G.A., Melis, M.P., Deiana, M., Dessi, M.A., and Corongiu, F.P., Conjugated linoleic acid and oxidative stress, *J. Am. Oil Chem. Soc.*, 75, 261–267, 1998.
- Becker, C.C. and Kyle, D.J., Developing functional foods containing algal docosahexaenoic acid, *Food Technol.*, 52, 687–670, 1998.
- Berdeaux, O., Voinot, E., Juaneda, P., and Sebedio, J.L., A simple method of preparation of methyl *trans*-10,*cis*-12- and *cis*-9,*trans*-11-octadecadienoates from methyl linoleate, *J. Am. Oil Chem. Soc.*, 75, 1749–1755, 1998.
- Bligh, E.G. and Dyer, W.J., A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol.*, 37, 911–917, 1959.
- Carrapiso, A.I. and Garcia, C., Development in lipid analysis: some new extraction techniques and *in situ* transesterification, *Lipids*, 35, 1167–1177, 2000.
- Christie, W.W., *Gas Chromatography and Lipids*, The Oily Press Ltd., Dundee, Scotland, 1989.
- Christie, W.W., Sebedio, J.L., and Juaneda, P., A practical guide to the analysis of conjugated linoleic acid, *Inform*, 12, 147–152, 2001.
- Cook, M.E. and Pariza, M., The role of conjugated linoleic acid (CLA) in health, *Int. Dairy J.*, 8, 459–462, 1998.
- Davis, A.L., McNeill, G.P., and Caswell, D.C., Analysis of conjugated linoleic acid isomers by ¹³C NMR spectroscopy, *Chem. Phys. Lipids*, 97, 155–165, 1999a.
- Davis, A.L., McNeill, G.P., and Caswell, D.C., Identification and quantification of conjugated linoleic acid isomers in fatty acid mixtures by ¹³C NMR spectroscopy, in *Advances in Conjugated Linoleic Acid Research*, Vol. 1, Yurawecz, M.P., Mossoba, M.M., Kramer, J.K.G., Pariza, M.W., and Nelson, G.J., Eds., AOCS Press, Champaign, IL, 1999b, pp. 164–179.
- Devitt, A.A., Latour, M.A., and Watkins, B.A., Modification of chicken egg-yolk lipids with conjugated linoleic acids, Abstracts 89th AOCS Annual Meeting, Chicago, IL, 1998, 22.

- Dobson, G., Identification of conjugated fatty acids by gas chromatography-mass spectrometry of 4-methyl-1,2,4-triazoline-3,5-dione adducts, *J. Am. Oil Chem. Soc.*, 75, 137–142, 1998.
- Dobson, G. and Christie, W.W., Structural analysis of fatty acids by mass spectrometry of picolinyl esters and dimethyloxazoline derivatives, *Trends Anal. Chem.*, 15, 130–136, 1996.
- Dobson, G., Christie, W.W., and Nikolova-Damyanova, B., Silver-ion chromatography of lipids and fatty acids, *J. Chromatogr. B*, 671, 197–222, 1995.
- Doyle, E., Scientific forum explores CLA knowledge, *Inform*, 9, 69–72, 1998.
- Enig, M.G., Rationale for lauric oils as physiologically functional foods for human immunodeficiency virus-infected individuals, 85th AOCS Annual Meeting, Atlanta, GA, *Inform*, 5(Abstr.), 471F, 1994.
- Eulitz, K., Yurawecz, M.P., Sehat, N., Fritsche, J., Roach, J.A.G., Mossoba, M.M., Kramer, J.K.G., Adlof, R.O., and Ku, Y., Preparation, separation, and confirmation of the eight geometrical *cis,trans* conjugated linoleic acid isomers 8,10- through 11,13–18:2, *Lipids*, 34, 873–877, 1999.
- Folch, J., Lees, M., and Stanley, G.H.S., A simple method for the isolation and purification of lipids from animal tissues, *J. Biol. Chem.*, 226, 497–509, 1957.
- Fritsche, J., Conjugated linoleic acid isomers in foods: physiological properties, analysis, contents and dietary intake, *Lipid Technol. Newsl.*, 4, 115–119, 1998.
- Fritsche, J. and Steinhart, H., Analysis, occurrence, and physiological properties of *trans* fatty acids (TFA) with particular emphasis on conjugated linoleic acid isomers (CLA) — a review, *Fett/Lipid*, 100, 190–210, 1998.
- Fritsche, J., Rickert, R., Steinhart, H., Yurawecz, M.P., Mossoba, M.M., Sehat, N., Roach, J.A.G., Kramer, J.K.G., and Ku, Y., Conjugated linoleic acid (CLA) isomers: formation, analysis, amounts in foods and dietary intake, *Fett/Lipid*, 101, 272–276, 1999.
- Garcia, D.J., Omega-3 long-chain PUFA nutraceuticals, *Food Technol.*, 52, 44–49, 1998.
- Garcia-Ayuso, L.E., Velasco, J., Dobarganes, M.C., and de Castro, M.D.L., Accelerated extraction of the fat content in cheese using a focused microwave-assisted Soxhlet device, *J. Agric. Food Chem.*, 47, 2308–2315, 1999.
- Gertz, Ch. and Fiebeg, H.-J., Determination of fat content by the Caviezel method (rapid method), *Eur. J. Lipid Technol.*, 102, 154–158, 2000.
- Gorski, D., 1997 Ingredient forecast. Product development for world markets, *Dairy Foods*, 98, 60–64, 1997.
- Ha, Y.L., Park, K.A., Kim, S.J., Park, C.W., and Park, S.J., Enhancement of oxidative stability of conjugated linoleic acid (CLA) by encapsulation in cyclodextrins, Abstracts 90th AOCS Annual Meeting, Orlando, Florida, 1999, S22.
- Haas, M.J., Kramer, J.K.G., McNeill, G., Scott, K., Foglia, T.A., Sehat, N., Fritsche, J., Mossoba, M.M., and Yurawecz, M.P., Lipase-catalyzed fractionation of conjugated linoleic acid isomers, *Lipids*, 34, 979–987, 1999.
- Haumann, B.F., Alternative sources for n-3 fatty acids, *Inform*, 9, 1108–1119, 1998.
- Hilliam, M., Functional foods: the western consumer viewpoint, *Nutr. Rev.*, 54, S189–S194, 1996.
- Hopkins, C.Y., Fatty acids with conjugated unsaturation, in *Topics in Lipid Chemistry*, Gunstone, F.D., Ed., Elek Science, London, 1972, pp. 37–87.
- Horrocks, L.A. and Yeo, Y.K., Health benefits of docosahexaenoic acid (DHA), *Pharmacol. Res.*, 40, 211–225, 1999.
- Hubbard, W.D., Sheppard, A.J., Newkirk, D.R., Prosser, A.R., and Osgood, T., Comparison of various methods for the extraction of total lipids, fatty acids, cholesterol, and other sterols from food products, *J. Am. Oil Chem. Soc.*, 54, 81–83, 1977.
- Iverson, J.L. and Sheppard, A.J., Determination of fatty acids in butter fat, using temperature-programmed gas chromatography of the butyl esters, *Food Chem.*, 21, 223–234, 1986.

- Jahreis, G., Fritsche, J., and Kraft, J., Species-dependent, seasonal, and dietary variation of conjugated linoleic acid in milk, in *Advances in Conjugated Linoleic Acid Research*, Vol. 1, Yurawecz, M.P., Mossoba, M.M., Kramer, J.K.G., Pariza, M.W., and Nelson, G.J., Eds., AOCS Press, Champaign, IL, 1999, pp. 215–225.
- Juaneda, P. and Sebedio, J.L., Combined silver-ion and reversed-phase high-performance liquid chromatography for the separation and identification of C₂₀ metabolites of conjugated linoleic acid isomers in rat liver lipids, *J. Chromatogr. B*, 724, 213–219, 1999.
- Kolanowski, W., Swiderski, F., and Berger, S., Possibilities of fish oil application for food products enrichment with ω -3 PUFA, *Int. J. Food Sci. Nutr.*, 50, 39–49, 1999.
- Kramer, J.K.G., Fellner, V., Dugan, M.E.R., Sauer, F.D., Mossoba, M.M., and Yurawecz, M.P., Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total *trans* fatty acids, *Lipids*, 32, 1219–1228, 1997.
- Kramer, J.K.G., Sehat, N., Fritsche, J., Mossoba, M.M., Eulitz, K., Yurawecz, M.P., and Ku, Y., Separation of conjugated fatty acid isomers, in *Advances in Conjugated Linoleic Acid Research*, Vol. 1, Yurawecz, M.P., Mossoba, M.M., Kramer, J.K.G., Pariza, M.W., and Nelson, G.J., Eds., AOCS Press, Champaign, IL, 1999, pp. 83–109.
- Lauritzen, D., Food enrichment with marine omega-3 fatty acids, *Food Ingredients*, No. 1/2, 41–44, 1994.
- Lavillonniere, F., Martin, J.C., Bougnoux, P., and Sebedio, J.-L., Analysis of conjugated linoleic acid isomers and content in French cheeses, *J. Am. Oil Chem. Soc.*, 75, 343–352, 1998.
- Lin, H., Boylston, T.D., Luedecke, L.O., and Schultz, T.D., Factors affecting the conjugated linoleic acid content of cheddar cheese, *J. Agric. Food Chem.*, 46, 801–807, 1998.
- Madsen, S., Development of a reduced-fat spread enriched with long-chain *n*-3 fatty acids, *Lipid Technol.*, 10, 129–132, 1998.
- McDonald, B.E. and Fitzpatrick, K., Designer vegetable oils, in *Functional Foods Biochemical and Processing Aspects*, Mazza, G., Ed., Technomic Publishing Company, Lancaster, PA, 1998, pp. 265–291.
- McDonald, R.E. and Mossoba, M.M., *Trans* fatty acids: labeling, nutrition, and analysis, in *Food Lipids and Health*, McDonald, R.E. and Min, D.B., Eds., Marcel Dekker, New York, 1996, pp. 161–197.
- Molkentin, J., Bioactive lipids naturally occurring in bovine milk, *Nahrung*, 43, 185–189, 1999.
- Morrison, W.R., Tan, S.L., and Hargin, K.D., Methods for the quantitative analysis of lipids in cereal grains and similar tissues, *J. Sci. Food Agric.*, 31, 329–340, 1980.
- Mossoba, M.M., Yurawecz, M.P., Kramer, J.K.G., Eulitz, K.D., Fritsche, J., Sehat, N., Roach, J.A.G., and Ku, Y., Confirmation of conjugated linoleic acid geometric isomers by capillary gas chromatography-Fourier transform infrared spectroscopy, in *Advances in Conjugated Linoleic Acid Research*, Vol. 1, Yurawecz, M.P., Mossoba, M.M., Kramer, J.K.G., Pariza, M.W., and Nelson, G.J., Eds., AOCS Press, Champaign, IL, 1999a, pp. 141–151.
- Mossoba, M.M., Kramer, J.K.G., Yurawecz, M.P., Sehat, N., Roach, J.A.G., Eulitz, K., Fritsche, J., Dugan, M.E.R., and Ku, Y., Impact of novel methodologies on the analysis of conjugated linoleic acid (CLA). Implications of CLA feeding studies, *Fett/Lipid*, 101, 235–243, 1999b.
- Muggli, R., Nutritional aspects of omega-3 long-chain polyunsaturated fatty acids, *Agro-Food-Industry Hi-Tech*, Jan./Feb., 35–36, 1997.

- Newton, I. and Snyder, D., Nutritional aspects of long-chain omega-3 fatty acids and their use in bread enrichment, *Cereal Foods World*, 42, 126–131, 1997.
- Nikolova-Damyanova, B., Momchilova, S., and Christie, W.W., Silver ion high-performance liquid chromatographic separation of conjugated linoleic acid isomers, and other fatty acids, after conversion to *p*-methoxyphenacyl derivatives, *J. High Resol. Chromatogr.*, 23, 348–352, 2000.
- Oomah, B.D. and Mazza, G., Flaxseed products for disease prevention, in *Functional Foods Biochemical and Processing Aspects*, Mazza, G., Ed., Technomic Publishing Company, Lancaster, PA, 1998, pp. 91–137.
- O'Shea, M., Lawless, F., Stanton, C., and Devery, R., Conjugated linoleic acid in bovine milk fat: a food-based approach to cancer chemoprevention, *Trends Food Sci. Technol.*, 9, 192–196, 1998.
- Ostrowska, E., Dunshea, F.R., Muralitharan, M., and Cross, R.F., Comparison of silver-ion high-performance liquid chromatographic quantification of free and methylated conjugated linoleic acids, *Lipids*, 35, 1147–1153, 2000.
- Padley, F.B., Gunstone, F.D., and Harwood, J.L., Occurrence and characteristics of oils and fats, in *The Lipid Handbook*, Gunstone, F.D., Harwood, J.L., and Padley, F.B., Eds., Chapman & Hall, London, 1994, pp. 47–223.
- Pariza, M.W., The biological activities of conjugated linoleic acid, in *Advances in Conjugated Linoleic Acid Research*, Vol. 1, Yurawecz, M.P., Mossoba, M.M., Kramer, J.K.G., Pariza, M.W., and Nelson, G.J., Eds., AOCS Press, Champaign, IL, 1999, pp. 12–20.
- Pariza, M.W., Park, Y., and Cook, M.E., Mechanisms of action of conjugated linoleic acid: evidence and speculation, *Proc. Soc. Exp. Biol. Med.*, 223, 8–13, 2000.
- Park, Y. and Pariza, M.W., Evidence that commercial calf and horse sera can contain substantial amounts of *trans*-10, *cis*-12 conjugated linoleic acid, *Lipids*, 33, 817–819, 1998.
- Ratnayake, W.M.N., Analysis of *trans* fatty acids, in *Trans Fatty Acids in Human Nutrition*, Sebedio, J.L. and Christie, W.W., Eds., The Oily Press Ltd., Dundee, Scotland, 1998, pp. 115–161.
- Rickert, R., Steinhart, H., Fritsche, J., Sehat, N., Yurawecz, M.P., Mossoba, M.M., Roach, J.A.G., Eulitz, K., Ku, Y., and Kramer, J.K.G., Enhanced resolution of conjugated linoleic acid isomers by tandem-column silver-ion high performance liquid chromatography, *J. High Resol. Chromatogr.*, 22, 144–148, 1999.
- Roach, J.A.G., Identification of CLA isomers in food and biological extracts by mass spectrometry, in *Advances in Conjugated Linoleic Acid Research*, Vol. 1, Yurawecz, M.P., Mossoba, M.M., Kramer, J.K.G., Pariza, M.W., and Nelson, G.J., Eds., AOCS Press, Champaign, IL, 1999, pp. 126–140.
- Roach, J.A.G., Yurawecz, M.P., Kramer, J.K.G., Mossoba, M.M., Eulitz, K., and Ku, Y., Gas chromatography-high resolution selected-ion mass spectrometric identification of trace 21:0 and 20:2 fatty acids eluting with conjugated linoleic acid isomers, *Lipids*, 35, 797–802, 2000.
- Schmidl, M.K., The role of lipids in medical and designer foods, in *Food Lipids and Health*, McDonald, R.E. and Min, D.B., Eds., Marcel Dekker, New York, 1996, pp. 417–436.
- Schuhardt, U. and Lopes, O.C., Tetramethylguanidine catalyzed transesterification of fats and oils: a new method for rapid determination of their composition, *J. Am. Oil Chem. Soc.*, 65, 1940–1941, 1988.
- Sebedio, J.L., Juaneda, P., Dobson, G., Ramilison, I., Martin, J.C., Chardigny, J.M., and Christie, W.W., Metabolites of conjugated isomers of linoleic acid (CLA) in the rat, *Biochim. Biophys. Acta*, 1345, 5–10, 1997.

- Sehat, N., Kramer, J.K.G., Mossoba, M.M., Yurawecz, M.P., Roach, J.A.G., Eulitz, K., Morehouse, K.M., and Ku, Y., Identification of conjugated linoleic acid isomers in cheese by gas chromatography, silver-ion high performance liquid chromatography and mass spectral reconstructed ion profiles. Comparison of chromatographic elution sequences, *Lipids*, 33, 963–971, 1998a.
- Sehat, N., Yurawecz, M.P., Roach, J.A.G., Mossoba, M.M., Kramer, J.K.G., and Ku, Y., Silver-ion high performance liquid chromatographic separation and identification of conjugated linoleic acid isomers, *Lipids*, 33, 217–221, 1998b.
- Sehat, N., Rickert, R., Mossoba, M.M., Kramer, J.K.G., Yurawecz, M.P., Roach, J.A.G., Adlof, R.O., Morehouse, K.M., Fritsche, J., Eulitz, K.D., Steinhart, H., and Ku, Y., Improved separation of conjugated fatty acid methyl esters by silver-ion high-performance liquid chromatography, *Lipids*, 34, 407–413, 1999.
- Shahidi, F. and Wanasundara, J.P.D., Extraction and analysis of lipids, in *Food Lipids Chemistry, Nutrition and Biotechnology*, Akoh, C.C. and Min, D.B., Eds., Mercel Dekker, New York, 1997, pp. 115–136.
- Shahidi, F., Functional seafood lipids and proteins, in *Functional Foods. Biochemical and Processing Aspects*, Mazza, G., Ed., Technomic Publishing Company, Lancaster, PA, 1998, pp. 381–401.
- Shantha, N.C., Decker, E.A., and Hennig, B., Comparison of methylation methods for the quantitation of conjugated linoleic acid isomers, *J. AOAC Int.*, 76, 644–649, 1993.
- Sim, J.S., Designer eggs and their nutritional and functional significance, *World Rev. Nutr. Diet.*, 83, 89–101, 1998.
- Simopoulos, A.P., Fatty acids, in *Designer Foods, Pharmafoods, Nutraceuticals*, Goldberg, I., Ed., International Thomson Publishing, New York, 1994, pp. 355–392.
- Spitzer, V., Marx, F., and Pfeilsticker, K., Electron impact mass spectra of the oxazoline derivatives of some conjugated diene and triene C₁₈ fatty acids, *J. Am. Oil Chem. Soc.*, 71, 873–876, 1994.
- Uauy-Dagach, R. and Valenzuela, A., Marine oils: the health benefits of *n*-3 fatty acids, *Nutr. Rev.*, 54, S102–S108, 1996.
- Ulberth, F. and Henninger, M., One-step extraction/methylation method for determining the fatty acid composition of processed foods, *J. Am. Oil Chem. Soc.*, 69, 174–177, 1992.
- Van Elswyk, M., Nutritional and physiological effects of flaxseed in diets for laying fowl, *World's Poult. Sci. J.*, 53, 253–264, 1997.
- Werner, S.A., Luedecke, L.O., and Schultz, T.D., Determination of conjugated linoleic acid content and isomer distribution in three cheddar-type cheeses: effects of cheese cultures, processing, and aging, *J. Agric. Food Chem.*, 40, 1817–1821, 1992.
- Willis, W.M., Lencki, R.W., and Marangoni, A.G., Lipid modification strategies in the production of nutritionally functional fats and oils, *Crit. Rev. Food Sci. Nutr.*, 38, 639–674, 1998.
- Wolff, R.L., Content and distribution of *trans*-18:1 acids in ruminant milk and meat fats. Their importance in Europeans diets and their effect on human milk, *J. Am. Oil Chem. Soc.*, 72, 259–272, 1995.
- Wolff, R.L., Bayard, C.C., and Fabien, R.J., Evaluation of sequential methods for the determination of butterfat fatty acid composition with emphasis on *trans*-18:1 acids. Application to the study of seasonal variations in French butters, *J. Am. Oil Chem. Soc.*, 72, 1471–1483, 1995.
- Wolff, R.L., Precht, D., and Molkenin, J., Occurrence and distribution of *trans*-18:1 acids in edible fats of natural origin, in *Trans Fatty Acids in Human Nutrition*, Sebedio, J.L. and Christie, W.W. Eds., The Oily Press Ltd., Dundee, Scotland, 1998, pp. 1–33.

- Yamasaki, M., Kishihara, K., Ikeda, I., Sugano, M., and Yamada, K., A recommended esterification method for gas chromatographic measurement of conjugated linoleic acid, *J. Am. Oil Chem. Soc.*, 76, 933–938, 1999.
- Yurawecz, M.P., Hood, J.K., Roach, J.A.G., Mossoba, M.M., Daniels, D.H., Ku, Y., Pariza, W., and Chin, S.F., Conversion of allylic hydroxy oleate to conjugated linoleic acid and methoxy oleate by acid-catalyzed methylation procedures, *J. Am. Oil Chem. Soc.*, 71, 1149–1155, 1994.
- Yurawecz, M.P., Roach, J.A.G., Sehat, N., Mossoba, M.M., Kramer, J.K.G., Fritsche, J., Steinhart, H., and Ku, Y., A new conjugated linoleic acid isomer, 7 *trans*, 9 *cis*-octadecadienoic acid, in cow milk, cheese, beef and human milk and adipose tissue, *Lipids*, 33, 803–809, 1998.
- Yurawecz, M.P., Kramer, J.K.G., and Ku, Y., Methylation procedures for conjugated linoleic acid, in *Advances in Conjugated Linoleic Acid Research*, Vol. 1, Yurawecz, M.P., Mossoba, M.M., Kramer, J.K.G., Pariza, M.W., and Nelson, G.J., Eds., AOCS Press, Champaign, IL, 1999, pp. 64–82.
- Yurawecz, M.P., Sehat, N., Mossoba, M.M., Roach, J.A.G., Kramer, J.K.G., and Ku, Y., Variations in isomer distribution in commercially available conjugated linoleic acid, *Fett/Lipid*, 101, 277–282, 1999.

3 Carotenoids and Provitamin A in Functional Foods

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3.1 INTRODUCTION

The definition of *functional food* can be very wide and variable, but we should not forget that all foods have at least one main function: alimentation. A functional food should be considered as a dietary ingredient that can have above-normal nutritional value. According to Gibson and Roberfroid (1995), a useful definition of functional food is *a dietary ingredient that affects its host in a targeted manner so as to exert positive effects that may, in due course, justify certain health claims*. This definition leads directly to the question: How can foods be made more functional? We might consider four possible mechanisms: (1) elimination of a component having a negative physiological effect; (2) increasing the concentration of components that contribute beneficial aspects; (3) addition of a new ingredient observed to have general advantages; and (4) partial substitution of a negative component by another, positive one, without adversely affecting the nutritional value of the food (Gibson and Fuller, 1998).

Current dietetic recommendations which tend to increase the consumption of fruits and vegetables are based on epidemiological evidence relating such diets with a longer and better-quality life. Fruits and vegetables are considered rich sources of antioxidants such as vitamin C, vitamin E, carotenoids, flavonoids, etc. However, they are not the only source of dietary antioxidants, and, for example, we can find high contents of vitamin E in nuts, seeds, and cereals and in eggs, margarines,

vegetable oils, and dairy products. Similarly, the carotenoids are not restricted to fruits and vegetables, so dairy products, vegetable oils, and some animal products can be considered an important source of these compounds (Strain and Benzie, 1998).

Research carried out in the last 70 years has shown that the carotenoid pigments present in fruits and vegetables are the main dietary source of vitamin A for most people, especially in poorer countries where vitamin A deficiency is a serious problem. β -Carotene is the main compound with provitamin A activity (Olson, 1993). When incorporated in the diet, it is broken down into two molecules of retinol (vitamin A) by action of the enzyme β -carotene-15,15'-dioxygenase in the intestine. However, β -carotene is not the only carotenoid with provitamin A activity, and, as we will discuss in detail, any carotenoid with at least one unsubstituted β -ring can undergo similar cleavages and give rise to a vitamin A molecule. Carotenoids such as α -carotene and β -cryptoxanthin can thus contribute substantially to the nutritional value of fruits and vegetables.

In recent years, however, growing evidence has indicated that this may not be the only contribution of carotenoids to health. Numerous epidemiological studies indicate that carotenoid-rich diets are correlated with a lower risk of contracting certain types of cancer, heart disease, and other important human diseases (Colditz et al., 1985; Mathews-Roth, 1982; Moon, 1989; Ziegler, 1989). While such facts by themselves do not show that the carotenoids are the active factors in this beneficial effect, experiments and tests with humans and other animals have shown that carotenoids do have a direct effect. Current attention is centered on the action of β -carotene as an antioxidant, as it may interfere in free radical oxidation (such as the peroxidation of lipids), typical of many degenerative diseases. Although it has been clearly demonstrated that β -carotene has a significant antioxidant effect *in vitro*, there is still no real proof that this is its *in vivo* function at the low concentrations in which it is found and under physiological conditions (Bendich and Olson, 1989; Burton and Ingold, 1984).

Recent research has demonstrated that the carotenoids can modify membrane structure and properties, with substantial effects on the human immune response system and on the process of gap-junction communication between neighboring cells (Bendich, 1989; Zhang et al., 1991). This opens up a range of exciting new possibilities of research and applications.

To be able to have any beneficial action, the carotenoids must be absorbed, transported, and deposited in certain tissues. All carotenes and many xanthophylls can be used, although it seems that the epoxyxanthophylls such as violaxanthin and neoxanthin, which are abundant in plants (particularly in green tissues), cannot be incorporated into and used by the human body. This brings up the important aspect of the bioavailability of dietary carotenoids, which depends on other dietetic factors, in particular the contribution of dietary fats. Carotenoids are absorbed more efficiently from cooked foods than from fresh ones, probably because cooking and processing liberates the carotenoids from their usual molecular environment, making them more accessible for solubilization. The carotenoids with (*Z*)-conformation are generally more bioavailable than the all-(*E*)-forms, apparently because the (*Z*)-forms have a lower tendency to produce microcrystalline aggregates and thus a higher solubility.

Furthermore, the carotenoid pigments, either in isolation or jointly with other natural pigments such as chlorophylls and anthocyanins, are responsible for food color. Color is the first characteristic the consumer perceives of a food, and it confers expectations of quality and flavor. Food quality is judged first on color, and the consumer will reject foods with an external color other than that established as correct. The food industry, knowing well this natural relation of color and quality (and vice versa), tries to adjust the industrial processes of transformation and preparation of foods to conserve the integrity of the compounds responsible for an acceptable color. This is not always possible, and it is normal practice to add coloring matter to enhance, homogenize, or even modify color to make the food more attractive to the consumer (Henry, 1996).

Carotenoids are thus compounds of great dietetic importance, not only as precursors of vitamin A, but also as molecules that take part in cell protection and consumer attraction. In consequence, carotenoid content and composition are important factors in the nutritional evaluation of fruits, vegetables, and foods in general (Britton and Hornero-Méndez, 1997). The latest advances show that all the carotenoids, not only β -carotene and others having provitamin A value, are of considerable benefit and should be included in the composition of any *functional food*. Current trends in legislation and the use of pigments or colorants in the food industry point toward the progressive exclusion of synthetic pigments in favor of natural ones, used not only for their coloring power, but also for the added value of contributing nutritional and physiological properties beneficial for health, such as in the case of carotenoids and anthocyanins. These aspects were discussed recently at the First International Symposium on Pigments in Food Technology (held March 24 to 26, 1999 in Seville, Spain), being the title of the opening lecture, *Pigments in Foods. More Than Just Colours*, a good reflection of the importance of the pigments in the food field (Britton, 1999).

3.2 INTRODUCTION TO THE CAROTENOIDS

Among all the pigments present in living organisms, there is no doubt that the carotenoids are the most widely distributed in nature. They are found throughout the plant kingdom — in both photosynthetic and non-photosynthetic tissues — in bacteria, in fungi, and in animals. The latter are unable to synthesize carotenoids, so they incorporate them from dietary plants. It is estimated that the annual production of carotenoids in nature is around 10^8 tons (Weedon, 1971).

In 1831, Wackenroder isolated an orange pigment from the carrot (*Daucus carota*) and coined the term “carotene” from the Latin word *carota*. Six years later, Berzelius assigned the name *xanthophylls* to the yellow pigment of autumn leaves. Nevertheless, it was not until 1906 that Tswett was able to separate the pigments from an extract of leaves, inventing chromatography. Around 1929, the works of von Euler, Karrer, and Moore demonstrated the relationship between the carotenoids and vitamin A, revealing their nutritional value. From then on, interest in these compounds has grown. Thanks to the continuous development of analytical techniques, the number of known naturally occurring carotenoids has risen from 11 in 1934 to 32 in 1948, 230 in 1971, 450 in 1987, and some 650 today (Eugster, 1995).

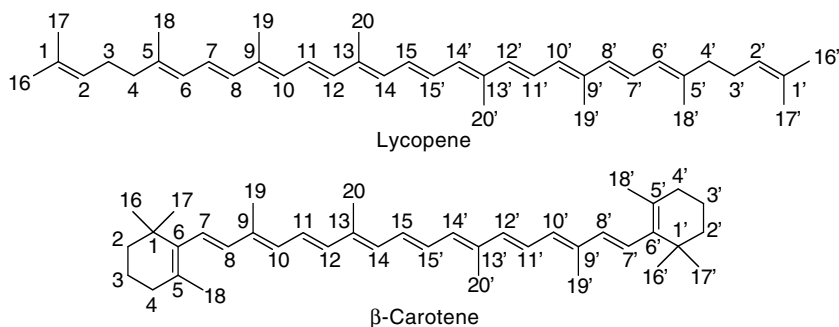


FIGURE 3.1 C_{40} skeleton and numbering scheme of carotenoids.

Most of the carotenoids found naturally in fruits and vegetables present a skeleton of 40 carbon atoms (C_{40}) and are biosynthesized from two molecules of an intermediary C_{20} (geranylgeranyl diphosphate), giving rise to a phytoene as generic precursor of the whole wide range of carotenoids present in the plant kingdom. The phytoene molecule undergoes a series of successive desaturations (up to four), introducing new double bonds into the carbon chain, resulting in spreading of the double bond conjugation and thus of the chromophore that is typical of these natural pigments and responsible for their chromatic properties. Successive structural changes, such as cyclization of one or both ends, hydroxylation, or introduction of other oxygenated functions, give rise to the great range of carotenoid structures found in nature.

In general, the carotenoids can be classified into two great groups: *carotenes*, which are strictly hydrocarbons, and *xanthophylls*, which are derived from the former and contain oxygenated functions. Structurally, the carotenoids may be acyclic (e.g., lycopene) or contain a ring of five or six carbons at one or both ends of the molecule (e.g., β -carotene). Figure 3.1 shows the structure and the system of numbering using lycopene and β -carotene as models of acyclic and bicyclic carotenoids, respectively. Figure 3.2 includes the structures of some representative carotenes and xanthophylls, and Appendix 3.I shows the structures of the carotenes and xanthophylls commonly found in foods.

Traditionally, trivial names have been given to new carotenoids after discovery, which in most cases refers to the natural source from which it had first been isolated, for example, zeaxanthin from maize (*Zea mays*) and lycopene from tomato (*Lycopersicon esculentum*). Because this system does not include any structural information, a semisystematic nomenclature was defined that does give structural information and additionally refers to the parent carotene. This system was approved by the International Union of Pure and Applied Chemistry (IUPAC), which published in 1975 a compendium of rules for the nomenclature of carotenoids (Britton, 1993; Weedon and Moss, 1995). Greek letters are used to designate the end groups that may be present in the carotenoid molecule. Those carotenoids lacking an end group are denominated *apocarotenoids*. Figure 3.3 shows the seven end groups that have been found in natural carotenoids, namely, β , ϵ , γ , κ , ϕ , χ , and ψ . In addition Table 3.1

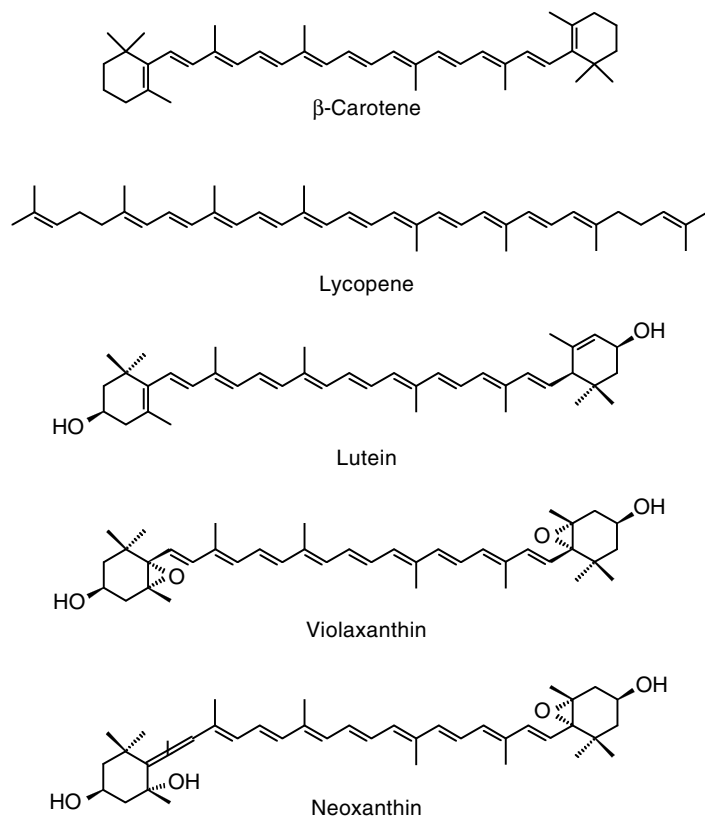


FIGURE 3.2 Some common carotenenes and xanthophylls.

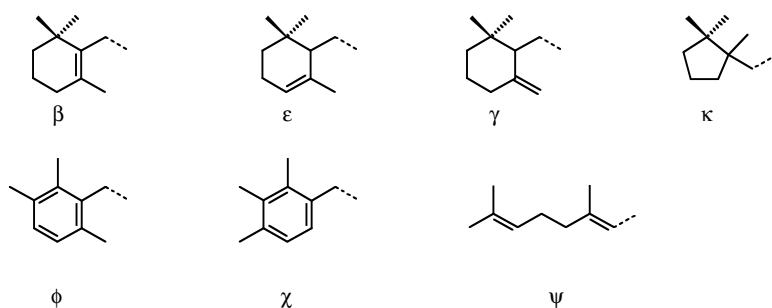


FIGURE 3.3 Chemical structures for the seven end groups found in natural carotenoids.

shows trivial and semisystematic names for the carotenenes and xanthophylls most commonly found in foods.

The presence of a large number of conjugated double bonds in the carotenoid molecule makes possible numerous geometric isomers [*(Z-E)*-isomers, also called *cis-trans*]. In practice, however, most of the carotenoids are naturally present as all-*(E)*. A few carotenoids are present in the *(Z)*-form in nature, such as bixin present

TABLE 3.1
Trivial and Semisystematic Names for Some Carotenes and Xanthophylls

Trivial Name	Semisystematic Name
Carotenes	
α -Carotene	β,ϵ -Carotene
β -Carotene	β,β -Carotene
δ -Carotene	ϵ,ψ -Carotene
γ -Carotene	β,ψ -Carotene
ϵ -Carotene	ϵ,ϵ -Carotene
ζ -Carotene	7,8,7',8'-Tetrahydro- ψ,ψ -carotene
Lycopene	ψ,ψ -Carotene
Neurosporene	7,8-Dihydro- ψ,ψ -carotene
Phytoene	7,8,11,12,7',8',11',12'-Octahydro- ψ,ψ -carotene
Phytofluene	7,8,11,12,7',8'-Hexahydro- ψ,ψ -carotene
Xanthophylls	
Antheraxanthin	5,6-Epoxy-5,6-dihydro- β,β -carotene-3,3'-diol
Astaxanthin	3,3'-Dihydroxy- β,β -carotene-4,4'-dione
Auroxanthin	5,8,5',8'-Diepoxy-5,8,5',8'-tetrahydro- β,β -carotene-3,3'-diol
Bixin	Methyl hydrogen 9'-(Z)-6,6'-diapocarotene-6,6'-dioate
Canthaxanthin	β,β -Carotene-4,4'-dione
Capsanthin	3,3'-Dihydroxy- β,κ -caroten-6'-one
Capsorubin	3,3'-Dihydroxy- κ,κ -carotene-6,6'-dione
Crocetin	8,8'-Diapocarotene-8,8'-dioic acid
α -Cryptoxanthin	β,ϵ -Carotene-3-ol
β -Cryptoxanthin	β,β -Carotene-3-ol
Cryptoxanthin-5,6-epoxide	5,6-Epoxy-5,6-dihydro- β,β -caroten-3-ol
Cucurbitaxanthin A	3',6'-Epoxy-5',6'-dihydro- β,β -carotene-3,5'-diol
Lactucaxanthin	ϵ,ϵ -Carotene-3,3'-diol
Lutein	β,ϵ -Carotene-3,3'-diol
Luteoxanthin	5,6,5',8'-Diepoxy-5,6,5',8'-tetrahydro- β,β -carotene-3,3'-diol
Mutatoxanthin	5,8-Epoxy-5,8-dihydro- β,β -carotene-3,3'-diol
Neochrome	5',8'-Epoxy-6,7-didehydro-5,6,5',8'-tetrahydro- β,β -carotene-3,5,3'-triol
Neoxanthin	5',6'-Epoxy-6,7-didehydro-5,6,5',6'-tetrahydro- β,β -carotene-3,5,3'-triol
Norbixin	6,6'-Diapocarotene-6,6'-dioic acid
Violaxanthin	5,6,5',6'-Diepoxy-5,6,5',6'-tetrahydro- β,β -carotene-3,3'-diol
Zeaxanthin	β,β -Carotene-3,3'-diol

in the annatto seeds (*Bixa orellana*) or pro-lycopene [a carotenoid with several double bonds and (Z)-configuration, poly-(Z)] present in certain varieties of tomato.

3.3 PRESENCE, DISTRIBUTION, LOCALIZATION, AND FUNCTIONS

Carotenoid pigments are widespread among living organisms, both plants and animals, but are found in greater concentration and variety in the former, which are the

only organisms (together with certain bacteria) able to biosynthesize them (Weedon, 1971). The distribution of carotenoids among the different groups of higher plants does not follow a single pattern. In green plant tissues, the class and content of carotenoid pigments follow the general model associated with the presence of chloroplasts, with β -carotene being the predominant carotene, followed by the xanthophylls, lutein, violaxanthin, and neoxanthin. Zeaxanthin, γ -carotene, β -cryptoxanthin, and antheraxanthin are found in small amounts. In the case of fruits, the xanthophylls are normally found in greater amounts. Exceptions are found in maize, the predominant pigments being lutein and zeaxanthin, while in mango (*Mangifera indica*) and persimmon (*Diospyros kaki*) they are β -cryptoxanthin and zeaxanthin. In contrast, in tomato the major carotenoid is lycopene, a carotene. In certain fruits, a carotenoid, besides being the major one, is limited totally or almost totally to a single plant species. Capsanthin and capsorubin are found almost exclusively in ripe fruits of the genus *Capsicum*, and are responsible for their attractive red color (Davies et al., 1970; Goodwin, 1976; Goodwin and Goad, 1970). The orange (*Citrus sinensis*) contains varying amounts of β -citraurin and β -citraxanthin (both apocarotenoids), together with β -cryptoxanthin, lutein, antheraxanthin, violaxanthin, and traces of their carotene precursors (Farin et al., 1983). The presence and distribution of the most common carotenoid pigments found in fruit and vegetables, and in general in foods, are shown in Table 3.2.

In green plant tissues, the xanthophylls are in the free form. However, as a consequence of leaf senescence and the ripening of many fruits, coinciding with the transformation of the chloroplasts into chromoplasts, the carotenoid pigments undergo esterification with different fatty acids. The esterification is related to the capacity of the plant (in particular fruits and flowers) to overproduce and accumulate carotenoid pigments. The change in esterification profile of the xanthophylls in fruits of red pepper has been proposed recently as a ripening index (Hornero-Méndez and Mínguez-Mosquera, 2000). The function of esterification, which does not affect the chromophore properties of the pigment, seems to be related in the case of fruits and flowers with attracting animals that act as the vehicle for the dissemination of seeds and pollen for increasing reproductive success (Bartley and Scolnick, 1995).

In the animal kingdom, carotenoids are incorporated via the diet and stored in different tissues. The egg yolk owes its yellow color to xanthophylls such as lutein and zeaxanthin and to traces of β -carotene. While the presence and distribution of carotenoids in mammals is very limited, other vertebrates, such as birds, fish, reptiles, and amphibians, and, above all, the invertebrates show a great diversity of carotenoid pigments and even have the capacity to modify structurally some of the carotenoids ingested in the diet (Schiedt, 1998).

In the case of the invertebrates, the carotenoids can be intimately associated to proteins, giving rise to a very important group of compounds known as carotenoproteins. Such association results in changes of the chromatic characteristics of the carotenoid, which presents coloration including green, blue, purple, and gray (Zagalsky, 1985). The presence of carotenoproteins in crustaceans has been known for some time, the first mention probably being that of the French biologist G. Pouchet in 1872. They are normally found in the exoskeleton and in the eggs and

TABLE 3.2
Natural Occurrence of Some Common Carotenes and Xanthophylls

Carotenoid	Natural Occurrence
Carotenes	
α -Carotene, β -carotene, δ -carotene, γ -carotene, ϵ -carotene, ζ -carotene	Fruits and vegetables, especially in carrots, sweet potato, and palm tree fruit; delta tomato mutant has δ -carotene as major carotene; rose hips are a good source for γ -carotene
Lycopene, neurosporene	Tomato (<i>Lycopersicon esculentum</i>), watermelon, and rose hips (<i>Rosa</i> spp.)
Phytofluene, phytoene	Carotenoid-rich fruits, flowers, and roots (carrot)
Xanthophylls	
Antheraxanthin	Anthers and petals of many yellow flowers; also in fruits and vegetables
Astaxanthin	Bird feathers, fish (salmon), and invertebrate animals (lobster, <i>Homarus gammarus</i>)
Bixin, norbixin	Annatto (<i>Bixa orellana</i>) seeds
Canthaxanthin	Mainly synthetic, naturally found in some cyanobacteria and green algae
Capsanthin, capsanthin-5,6-epoxide, capsorubin	<i>Capsicum annuum</i> ripe fruits
Crocetin	Saffron (<i>Crocus sativus</i>) flowers
Cucurbitaxanthin A	Pumpkin (<i>Cucurbita maxima</i>) flesh
Lactucaxanthin	Lettuce (<i>Lactuca sativa</i>) leaves
Lutein, violaxanthin, neoxanthin, mutatoxanthin (as minor carotenoid)	Green fruits, vegetables, and flowers
Luteoxanthin, neochrome, auroxanthin	Vegetables and fruits processed under acid conditions and fermentation
Rubixanthin	Rose hips (<i>Rosa</i> spp.)
Zeaxanthin, β -cryptoxanthin, α -cryptoxanthin, cryptoxanthin-5,6-epoxide	Seeds (corn), flowers, and fruits: mango, papaya, and persimmon

ovaries, suggesting their participation in the animal's development and in the nutrient reserve. An important function associated to the presence of carotenoproteins is protective coloring, used as a means of camouflage in the environment (cryptic function) or as a form of protection from the harmful effects of external agents such as radiation (Lee, 1977).

In plants, the carotenoids are located and accumulated in specialized subcellular organelles called plastids, namely, chloroplasts and chromoplasts (Goodwin and Britton, 1988). The chloroplasts are present in all photosynthetic tissues (mainly leaves), where practically all the carotenoids are present in the form of chlorophyll-carotenoid-protein complexes (photosystems) at the level of the thylakoid membranes. In this environment, the carotenoids have their prime natural function as

assistant collectors of light energy (antenna pigments) in the photosynthetic process, since, due to their absorption spectrum, they are able to capture photons that escape the reach of the chlorophylls. Nevertheless, the chromoplasts present in flowers, ripe fruits, and certain roots and tubercles are the organelles specializing in the massive accumulation of carotenoids and having the greatest variety of structural forms. In the case of the chromoplasts, the carotenoids are usually accumulated in lipid-rich structures, the plastoglobules, as, for example, in the fruits of the genus *Capsicum* and many flowers. In certain cases, such as tomato, carrot (*Daucus carota*), and pumpkin (*Cucurbita maxima*), the presence of carotenoid crystals has also been reported, mainly carotenes, immersed in the stromatic space (Sitte et al., 1980). The change from chloroplast to chromoplast, which is associated with the fruit ripening process, is especially important in the case of the fruits denominated *carotenogenic* (for example, pepper and tomato), characterized by a massive synthesis of carotenoids during ripening, which is usually accompanied by a change in the carotenoid profile of the fruit. It is noteworthy that the chromoplast xanthophylls are usually esterified with different fatty acids, increasing their lipophilic character and facilitating their accumulation in the plastoglobules. In the case of fruits and flowers, the main function of carotenoids is undoubtedly to attract animals (insects, birds, and mammals) so that they co-operate in seed dispersion and pollen transport (Bartley and Scolnik, 1995).

The carotenoids also play a very important role as protectors of the chlorophylls and the photosynthetic apparatus in general by blocking (the quenching effect) very reactive forms of triplet chlorophylls (^3Chl) and singlet oxygen ($^1\text{O}_2$) formed during the capture of light energy. The carotenoids also take an active part in the plant's photoprotective and antioxidant action (Frank and Cogdell, 1993).

3.4 GENERAL PROPERTIES

The carotenoids are lipophilic substances and thus insoluble in aqueous medium, except in certain cases where highly polar functional groups are present, as in norbixin, a carotenoid with dicarboxyl acid structure. The presence of the long, extensive system of conjugated double bonds (or polyene chain) is responsible for one of the most distinctive characteristics of the carotenoids: light absorption. A chromophore with seven or more double bonds gives the capacity of absorbing light in the visible range and, consequently, the observation of colors spanning from yellow to red via a great variety of orange tones. Moreover, the polyene chain makes the carotenoid molecule extremely susceptible to isomerizing and oxidizing conditions such as light, heat, or acids. These properties regarding its structural lability largely determine the mode of operation and the precautions to be taken when working on the isolation and identification of carotenoids in the laboratory.

The properties of carotenoid pigments *in vitro* — that is, once extracted and dissolved — may be different to those *in vivo* because of the interaction with the physicochemical environment (mainly lipids and proteins) surrounding the pigments. This can be particularly critical regarding the functionality and action of the carotenoids *in vivo*.

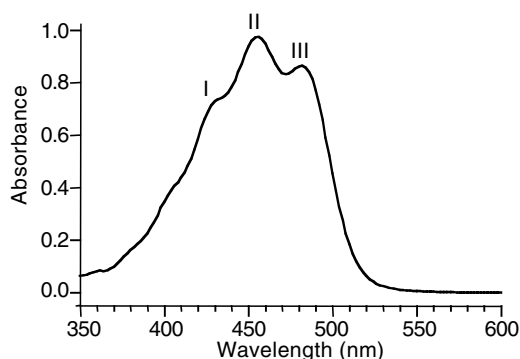


FIGURE 3.4 General ultraviolet-visible light absorption spectrum of carotenoids.

3.5 SPECTROSCOPIC PROPERTIES

The characteristic visible light absorption spectrum of the carotenoid pigments is due to the system of conjugated double bonds of their hydrocarbon chain (polyene). For a given carotenoid, the positions of the bands of maximum light absorption ($\lambda_{\max}$) are a function of the number of conjugated double bonds in the molecule (Figure 3.4). The absorption maxima are usually referred to using Roman numerals (I, II, III). The introduction of a new conjugated double bond into the chromophore causes a bathochromic displacement (to higher wavelength) of 20 to 22 nm in the absorption maxima ($\lambda_{\max}$), although this effect may be modified by the presence of different end groups. If the end group is a β -ring, the contribution to the chromophore is only 9 to 11 nm. In the case of an ϵ -ring, the conjugation of its double bond is lost and it does not participate in the chromophore. If the double bond of the β -ring is replaced by a 5,6-epoxide group, there is a hypsochromic displacement of 6 to 9 nm in $\lambda_{\max}$. The conversion in acid medium of the 5,6-epoxide group to 5,8-epoxide causes a new hypsochromic displacement of 20 to 22 nm because of the loss of a conjugated double bond at positions 7 and 8. The introduction of a hydroxyl group into the cyclic end group (normally positions 3 and 3') has almost no effect on the position of the absorption maxima, and the same happens with ketone groups not conjugated with the polyene chain, whereas the conjugated ones cause a bathochromic displacement of 5 to 10 nm in the maxima. Figure 3.5 illustrates some of these changes in the electron absorption spectrum depending on the nature of the chromophore. The (*Z/E*)-isomerism has a profound effect on the absorption spectrum, with a new maximum appearing in the near ultraviolet (UV) zone, around 320 to 340 nm.

The shape of the absorption spectrum of the carotenoid, and the positions of the absorption maxima, can vary depending on the interactions of the molecule with the solvent or lipid environment in which it is dissolved (Britton, 1995a). In general, solvents with low polarity have little effect on the position of the absorption maxima, so that for a given carotenoid, the values of $\lambda_{\max}$ are almost identical in hexane, light petroleum, diethyl ether, methanol, and ethanol. Acetone, commonly used in carotenoid extraction, causes a bathochromic displacement of around 2 to 6 nm in the

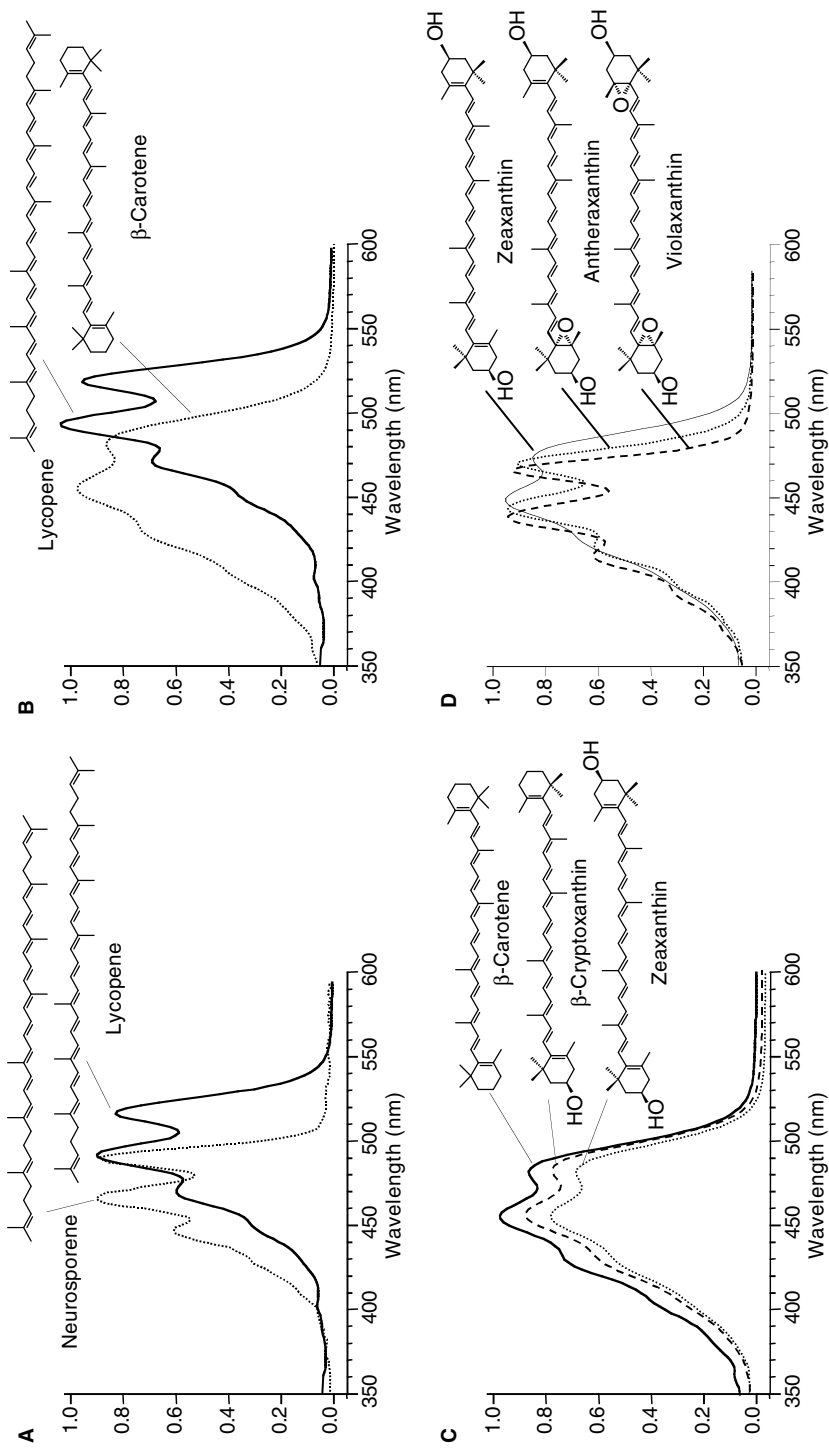


FIGURE 3.5 Effect of some structural changes of the chromophore on the UV-visible light absorption spectrum: (A) increase of the chromophore length, (B) cyclization, (C) hydroxylation, and (D) epoxidation.

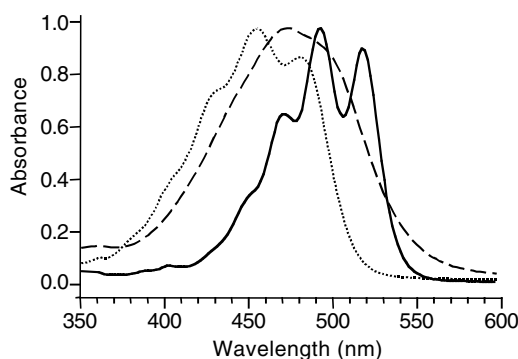


FIGURE 3.6 Effect of structure and end groups on the spectral fine structure. Lycopene (—), β -carotene (.....), and capsanthin (- -).

maxima compared with the aforementioned. In contrast, very polar solvents such as chloroform, benzene, and pyridine cause very significant bathochromic displacements (10 to 25 nm), which are extreme in the case of carbon disulfide (30 to 40 nm).

The acyclic carotenoids usually present greater persistence or a finer structure than the cyclic (monocyclic and bicyclic) ones. This is related to the noncoplanarity of the end rings with the central polyene chain. The first of the absorption maxima in carotenoids with two β -end groups, for example, β -carotene, appears as an inflexion. Most of the ketocarotenoids, such as capsanthin, where the carbonyl group is conjugated with the polyene chain, lose the fine structure, and only a wide main band is observed with very weak inflexions on each side (Goodwin, 1980). Figure 3.6 compares the fine structure of the UV-visible spectra for various carotenoids. When comparing the absorption spectrum of a given carotenoid, it is important to compare not only the positions of the absorption maxima ($\lambda_{\max}$), but also the shape and fine structure (defined by % III/II).

Carotenoid pigments also present other spectroscopic properties such as fluorescence and absorption of energy in the infrared region (IR). Fluorescence is a property rarely present in the carotenoids, and, in fact, only a few carotenoids fluoresce when they are excited at appropriated wavelengths (e.g., phytofluene). Therefore, fluorescence spectroscopy is not frequently used in carotenoid studies. Similarly, the use of IR spectroscopy is restricted essentially to the identification of functional groups, mainly hydroxyle, carbonyle, and allene.

3.6 NUTRITIONAL AND BENEFICIAL PROPERTIES OF THE CAROTENOIDS

Nutritionally, the main physiological function of the carotenoids is their capacity as precursors of vitamin A; therefore, they are said to have provitamin A value. This important quality has led some authors to propose their classification according to nutritional activity (depending on their provitamin A character) and their biological activity (anti-ulcer, anti-cancer, immunological regulators, antenna photosynthetic pigments, etc.) (Bendich and Olson, 1989; Swanson and Parker, 1996).

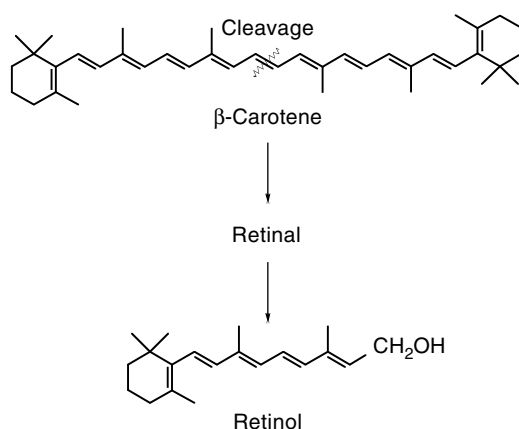


FIGURE 3.7 Central enzymatic cleavage of a β -carotene molecule to give two molecules of vitamin A.

TABLE 3.3
Relative Provitamin A Activity of Some
Representative Carotenes and Xanthophylls

Carotenoid	Activity (%)
All- <i>trans</i> - β -carotene	100
9- <i>cis</i> - β -Carotene	38
13- <i>cis</i> - β -Carotene	53
All- <i>trans</i> - α -carotene	53
9- <i>cis</i> - α -Carotene	13
13- <i>cis</i> - α -Carotene	16
All- <i>trans</i> -cryptoxanthin	57
9- <i>cis</i> -Cryptoxanthin	27
15- <i>cis</i> -Cryptoxanthin	42
β -Carotene-5,6-epoxide	21
Mutatochrome	50
γ -Carotene	42–50
β -Zeacarotene	20–40

Note: Data from Bauernfeind, 1972; Zechmeister, 1949.

The condition for a carotenoid to have such activity is that it possesses at least one unsubstituted end group with a β -ring. β -Carotene presents the greatest potential activity, since the central enzymatic cleavage of its molecule originates two molecules of vitamin A (Figure 3.7). Other carotenes such as α -carotene, γ -carotene, β -apo-8'-carotenal, and β -cryptoxanthin give rise to only one molecule of vitamin A, as they possess only one β -ring in their structure. Table 3.3 shows the relative provitamin A activity of some carotenoids.

The conversion of carotenoid to retinol takes place in the intestinal mucosa by action of the enzyme β -carotene-15,15'-dioxygenase on β -carotene, giving rise to two molecules of retinal, subsequently reduced to retinol (vitamin A), which is esterified with long-chain fatty acids, transported, and stored in the liver. Although one molecule of β -carotene can be metabolized into two of retinol, the *in vitro* assays carried out in 1967 by the FAO/WHO established that only one half of the β -carotene is converted to retinol and only one third of the carotenoid is absorbed in the intestine; therefore, one sixth of the β -carotene ingested is metabolically available as vitamin A (FAO/WHO, 1967). The term "retinol equivalent" (RE) was introduced to express the content in vitamin A (1 RE = 6 μ g β -carotene).

In humans and in mammals, in general, it has been shown that the carotenoids ingested in the diet are partly absorbed as such and are deposited in various tissues, such as adipose and plasma, and in the macula, where lutein and zeaxanthin have been found. The highest concentration of carotenoids is found in the plasma, always associated to lipoproteins and mainly to the low-density fraction [low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL)]. The carotenoids most commonly found in the plasma are α -carotene, β -carotene, lycopene, zeaxanthin, lutein, canthaxanthin, and β -cryptoxanthin (Clevidence and Bieri, 1992; Krinsky et al., 1958).

Besides being provitamin A precursors, the carotenoid pigments present a functional property; they are liposoluble antioxidants (Britton, 1995b; Krinsky, 1988). This property is not linked with the provitamin A nature, and all the carotenoid pigments can potentially develop it. This is because most have a carbonated central polyene structure responsible for the antioxidant nature of these compounds.

There are various factors in the effectiveness of the antioxidant action. Among these are the presence of oxygenated functional groups in the structure of the pigment (Terao, 1989), the conditions of the medium where the pigment acts (Liebler, 1993; Martin et al., 1999), and the nature of the prooxidant substance (Everett et al., 1996). Any of these factors may cause a self-oxidizing effect in place of the expected antioxidant beneficial one. Nevertheless, different *in vitro* and *in vivo* studies have concluded that the antioxidant action of pigments such as β -carotene and lycopene is effective.

Other health-benefiting effects of the carotenoid pigments are derived from their antioxidant action, which can protect against certain cancers and tumors related with the appearance of free radicals (prooxidant substances). By intercepting these harmful substances, the carotenoid pigments become chemiprotectors or anticancerigenic substances. Numerous recent epidemiological studies have shown the positive relationship between the level of lycopene ingested in the diet and the lower probability of the appearance of prostate cancer (Gann et al., 1999; Johnson, 2000; Stahl and Sies, 1996). Any other process involving prooxidant substances (cell aging, appearance of ulcers, etc.) can be attenuated by this carotenoid function (Handelman, 1996).

An important aspect is bioavailability, which is defined as the carotenoid fraction ingested that is available for use under normal physiological conditions or for storage. As mentioned before, the assimilation of carotenoid pigments involves their absorption, transport, and metabolization. During this complex process, part of the ingested carotenoids is lost from and another fraction is incorporated into the cell

structure where the function takes place. A primordial factor in bioavailability is the liposoluble nature of these substances, obviously important in the absorption and transport stages (Van het Hof et al., 2000). Carotenoid absorption and transport are reduced when fat consumption is low, so a minimum fat consumption is required to increase the absorption and subsequent transport of carotenoids. The consumption of fiber leads to a decreased absorption of fats and liposoluble substances and a decreased bioavailability of carotenoids (Castenmiller and West, 1998).

The presence of oxygenated functional groups also modifies the bioavailability of these compounds. It has been demonstrated recently that some ketocarotenoids are more rapidly absorbed and metabolized than other carotenes such as, for instance, lycopene (Oshima et al., 1997). These xanthophylls do not present provitamin A activity, but their antioxidant action is more effective than that of β -carotene. The incorporation of the carotenoid pigments into cell structures is affected by the pigment structure and the presence of functional groups that may modify the interaction with other molecules. Such structure, as mentioned previously, determines the effectiveness of the pigment's action.

3.7 USES OF CAROTENOID PIGMENTS

3.7.1 CAROTENOID AS FOOD COLORANTS

The attractive colors of carotenoid pigments, ranging from yellow to deep red, make the fruits that contain large amounts of them a good raw material for the natural colorant processing industry. Pigment extracts obtained from natural sources are used to enhance, correct, or contribute the color of foods. This is of special importance because consumer criteria of an ideal product are based on its organoleptic properties, among which color is a prime indicator of quality (Henry, 1996).

The use of food colorants, including carotenoids, is the subject of legislation, with compulsory rules that are more or less restrictive from country to country. In the European Union, current legislation lays special emphasis on the protection of consumer interests and health, promoting the use of natural pigments. In the case of the carotenoids, their use is permitted in all those foods in which the addition of colorants is permitted. Table 3.4 shows the carotenoids most commonly used and the code assigned that must appear on the product label. In the United States, legislation does not require certification for certain natural colorants, including the carotenoids in isolated form and various preparations or extracts rich in them. Table 3.5 shows carotenoid-rich colorants that are currently permitted by the U.S. Food and Drug Administration (FDA) (Downham and Collins, 2000).

The advantage of using carotenoids rather than other substances as colorants is their natural origin, which neutralizes any rejection (especially on the part of the consumer) when they are used to color foods. The external addition of carotenoids to foods achieves a triple effect. The first is the color given. Second, the nutritional value of the food increases if pigments with provitamin A activity are added. Third, most carotenoids increase the liposoluble antioxidant content. Of the three effects, the most important for the industry is the first, but the benefits of the other two should always be kept in mind.

TABLE 3.4
Natural Carotenoids (or Nature-Identical Forms)
Listed by the European Union and Approved
for Use in Foods

Colorant Code	Pigment Composition
E160a	β -Carotene and mixtures of carotenes
E160b	Annatto, bixin, norbixin
E160c	Paprika extract (oleoresin), capsanthin, capsorubin
E160d	Lycopene
E160e	β -Apo-8'-carotenal
E161b	Lutein
E161g	Canthaxanthin

Note: Data from Henry, 1996.

TABLE 3.5
Natural Carotenoids (or Nature-Identical Forms) and
Rich Carotenoid Sources Listed by the FDA for Food
and Beverage Use

Annatto extract
 β -Apo-8'-carotenal
 β -Carotene
Canthaxanthin
Carrot oil
Paprika and paprika oleoresin
Saffron

Note: Data from Henry, 1996.

The liposoluble nature of these compounds determines, *a priori*, the type of food in which they can be incorporated to dissolve efficiently. β -Carotene is added to fatty foods such as butter, cheeses, and oils, although other pigments such as bixin and apocarotenals are also employed. Paprika and oleoresins industrially obtained from red pepper are used directly or as an ingredient in the manufacture of sauces and meat products. Saffron, which contains crocin as a major pigment (a diester of crocetin with the disaccharide gentiobiose), is used as a hydrosoluble condiment for soups and to color foods and drinks. Other hydrosoluble preparations of β -carotene and other pigments such as canthaxanthin and apocarotenoids are used to color drinks. Norbixin, a product derived from the saponification of bixin, is hydrosoluble and is used to color ice cream, cereals, and cheese.

The addition of carotenoids to the diet of different animals is commonly used as a method to incorporate certain carotenoids into products that will be obtained

from such animals. For instance, β -carotene is added to cattle foodstuff to increase the concentration of provitamin A in milk. In poultry, alfalfa and maize, respectively, are used as lutein- and zeaxanthin-rich sources, pigments that are incorporated for the coloration of the skin and, in particular, egg yolk. The red and yellow coloring of the feathers of certain birds is due to the presence of dietary carotenoids. In the case of salmon, the red color of the flesh is due to pigmentation with dietary astaxanthin and canthaxanthin, which can be introduced artificially in animals bred on fish farms.

3.7.2 OTHER USES: PHARMACEUTICALS, CLINICAL, AND COSMETIC

The main use of carotenoids in drugs is to correct the levels of vitamin A in patients with hypovitaminosis or requiring an extra supply of vitamins. However, other applications have been developed not related with the provitamin A character, but rather with photochemical and antioxidant properties. For many years, these pigments have been used in therapy to reduce the effects of erythropoietic protoporphyria, a skin disease related with metabolism of the porphyrins. Carotenoids have also been considered as protectors in certain cancer treatments, especially those requiring radiotherapy. Carotenoid pigments are used in cosmetic products in the form of suspensions, emulsions, or lotions; in lipsticks; and in makeup foundations.

3.8 INDUSTRIAL PRODUCTION OF CAROTENOIDS

The first synthetic carotenoid was β -carotene, produced on an industrial scale since 1954, following the patent of Hoffmann-La Roche. Since then, the synthesis of carotenoids for their sale and use as food colorants has reached an annual production of 500 tons, with a value of \$300 million (U.S. dollars) (Britton et al., 1995).

Six main carotenoids are produced industrially by chemical synthesis: β -carotene, canthaxanthin, astaxanthin, β -apo-8'-carotenal, β -apo-8'-carotenoic ethyl ester, and citranaxanthin. The pigment is usually presented in microcrystalline form for its use in fatty foods or microencapsulated as a hydrophilic colloid for use in aqueous media.

Current trends in industrial production are toward the introduction of environmentally safer biotechnological processes using microorganisms bacteria (*Flavobacterium multivorum* and *Brevibacterium linens*), yeasts (*Phaffia rhodozyma*), fungi (*Phycomyces*), and microalgae (*Haematococcus pluvialis* and *Dunaliella salina*) for carotenoid production at an industrial scale. Recent developments in the molecular biology of carotenoid biosynthesis have provided a variety of genes that can be employed, once introduced in a proper host organism, for the biotechnological production of selected carotenoids (Armstrong, 1994; Bartley and Scolnik, 1994; Sandmann et al., 1999; Scolnik and Bartley, 1996)

3.9 ANALYSIS OF CAROTENOIDS IN FOODS

3.9.1 INTRODUCTION

The high number of carotenoid pigments in nature (more than 650) and their structural variability make it practically impossible to describe a general methodology

for their analysis. Fortunately, the number of carotenoid pigments in the food field is relatively small, even though their complexity remains.

For many years, the analysis of carotenoids in foods has focused on the determination of the provitamin A value (Rodríguez-Amaya, 1989). Many of the existing data are based on a poor identification and structural assignation of the pigments, causing great errors in the calculation of provitamin A. Calculation from the total carotenoid content of a food overestimates the provitamin A value. On the other hand, underestimation is frequent when only β -carotene is considered as the provitamin A source. Currently, it is clear that the nutritional or physiological *functionality* of the carotenoids goes beyond provitamin A activity, so those carotenoids qualified as and long considered inactive (from the provitamin point of view) should not be qualified as *afunctional*.

The continuous advances in instrumental techniques for organic compound analysis enable us to be rigorous in the analysis of carotenoid pigments. The following sections describe the main stages in the procedures of extraction, isolation, identification, and quantification of carotenoid pigments in foods of plant and animal origin.

3.9.2 GENERAL PRECAUTIONS

Work with carotenoid pigments is based on a series of general principles and strategies, which in practice can be modified depending on the work to be carried out, on the techniques to be applied, and, above all, on the raw material. In any case, the existence of an extensive polyene chromophore makes the carotenoids highly sensitive to heat, oxygen, light, and, in some cases, acids and alkalis. This means that the precautions taken with other natural products have to be stretched to the maximum when working with carotenoid pigments. Whenever possible, a quick and careful manipulation will minimize possible losses from destruction and the appearance of artifacts.

The oxidative degradation of carotenoids in the presence of molecular oxygen, and in general of any oxidant species, and the potentiation of their effects when combined with light and/or heat will indicate the main precautionary measures to be taken. Whenever possible, samples from the extraction of carotenoids should be stored *in vacuo* or under inert atmosphere (Ar or N₂). In order to minimize structural isomerizations during isolation and chromatographic analysis of the pigments, an essential precaution is their protection from heat and light, especially the latter when the sample includes photosensitizing substances (e.g., chlorophylls). Similarly, the sample should not be subjected to excessive heat, so the use of solvents with a high boiling point is generally inadvisable when evaporation is envisaged.

Practically all the carotenoids undergo dehydration, isomerization, and finally decomposition when subjected to acid action. Isomerization by acid action is shown particularly in the case of carotenoids with 5,6-epoxide groups, which are quantitatively transformed to 5,8-epoxide. Pigments containing 5,6-epoxide groups, such as antheraxanthin, neoxanthin, and violaxanthin, are widely distributed in plants, which also contain organic acids in amounts that vary, though are always enough to cause isomerization to 5,8-epoxide. The appearance of such artifacts is usually prevented

by adding neutralizing agents, such as sodium bicarbonate (NaHCO_3), during the extraction and disruption of the plant material. Whenever possible, the use of organic solvents which may contain traces of acids is avoided, such is the case of chloroform which usually contains a small amount of hydrogen chloride (HCl).

In contrast, most carotenoids are considerably stable to the action of alkalis, enabling saponification as a routine procedure to eliminate fatty matter and chlorophylls, or for hydrolysis of the esters of xanthophylls with fatty acids. Certain carotenoids are, however, alkali sensitive, notably, astaxanthin and, in general, the carotenoids containing 3-hydroxy-4-oxo- β -type end groups. When analyzing samples containing such carotenoids, contact with alkalis should be avoided.

3.9.3 EXTRACTION

The natural ubiquity of carotenoids in both the animal and plant kingdoms means that there is an enormous variety of sources and materials containing them, with very different characteristics. Consequently, no single, universally applicable extraction method can be established. In each case, the extraction system must be adapted to the characteristics of the tissue or source from which the pigments will be extracted, always carefully observing the general precautions required in the analysis of that type of substance, especially with regard to its photo- and thermolability and, if applicable, to the presence of acids and/or alkalis.

3.9.3.1 Preparation of the Sample

The sample to be analyzed should have been taken as recently as possible and is damage free to ensure that the pigment fraction has not been modified. If the analysis is not going to be performed immediately, the sample should be stored in the refrigerator or even frozen (at -30°C) for prolonged storage periods. In samples with a high content in water, lyophilization may be useful to remove the water, rehydrating with a small amount of the same in the later extraction step. If the sample is already lyophilized or dehydrated, it will also have to be rehydrated for the extraction.

The sample should be as representative as possible, with the removal of any damaged material and those tissues either not containing pigmentation or whose presence might interfere in the analysis. The weight of sample for analysis will depend on the carotenoid content. For samples having high carotenoid concentration, 2 to 3 g are usually taken, increasing to 10 g when the water content is high.

3.9.3.2 Choice of Solvent and Extraction

Because of the lipophilic nature of the carotenoids and because most foods contain a certain amount of water, the organic solvent used for the extraction must be miscible with water, as are, for example, methanol and ethanol. After one or two extractions with the solvent, the material to be extracted can be treated with another organic solvent (acetone, THF, hexane, diethyl ether) not necessarily miscible with water.

When the material to be extracted does not contain a large amount of fat, the number of possible extraction solvents is higher. The most frequent solvents are

acetone, methanol, ethanol, mixtures of these, and even mixtures with water (acetone–water, 80:20).

A high fat content can interfere in the later stages of analysis and must be removed. This is done directly by saponification (using KOH–methanol 20% (w/v)) or by using phase distribution techniques with two solvents, one lipophilic and the other selectively retaining the pigments. The phase distribution is carried out after the homogenization and filtration stage described next.

To facilitate contact between the solvent and material to be extracted, homogenization of the sample using appliances is advisable in all cases. Traditionally, this was done in a mortar with sand, disgregating and homogenizing the sample with the extraction solvent. More sophisticated and better are the *Ultra-Turrax* or *Polytron* homogenizers, which give a greater disgregation of the material. The addition of NaHCO₃ during this stage helps to neutralize acids liberated during disgregation and their harmful effect on the carotenoids. The extract obtained can be filtered *with vacuo* or centrifuged, and the residue can be collected to continue its extraction until colorless.

3.9.3.3 Removal of Fatty Matter and Final Preparation of the Extract

Any high lipid content present after the extraction stage must be removed. Two techniques are available: phase distribution and saponification. Phase distribution is particularly useful when analyzing chlorophylls, which are destroyed by alkali action, so that saponification cannot be used to remove the fatty matter. The same technique is necessary when the sample contains alkali-sensitive carotenoid pigments (astaxanthin and bixin) and when studying xanthophyll esters present in ripe fruits having a high lipid content. An example of phase distribution is that used for the analysis of chlorophylls and carotenoids in extracts obtained from olives (*Olea europaea*) or their oils, although it can also be used in many other samples having high fat content (Mínguez-Mosquera and Garrido-Fernández, 1985; Mínguez-Mosquera et al., 1991a). The solvents of extraction and distribution used are *N,N*-dimethylformamide and hexane, both immiscible. The hexane phase recovers the carotenes and fats, and the *N,N*-dimethylformamide phase recovers the xanthophylls and chlorophylls.

Saponification is the technique most used for the removal of fatty matter and other components such as chlorophylls (when their analysis is not required). In addition, saponification hydrolyzes the fatty acid esters of xanthophylls present in many ripe fruits, facilitating subsequent stages of analysis (such as isolation, identification, and quantification). The general procedure of pigment extract saponification is usually preceded by a step of transfer to diethyl ether, which is immiscible with water and has a low boiling point (below 35°C), simplifying water removal and its own removal by evaporation. This transfer not only helps saponification, but also prevents the formation of saponification artifacts, especially by reaction between ketones (usually due to the presence of acetone in the extract) and apocarotenal aldehyde groups.

Transference is carried out by adding a sufficient amount of diethyl ether to the extract and shaking in a decanting funnel, followed by the addition of aqueous 10%

(w/v) NaCl solution. Two clearly different phases are obtained: the ether phase (containing the pigments) and the aqueous phase. The ether phase is washed with an aqueous solution of 2% (w/v) Na_2SO_4 to remove traces of water. Saponification is carried out by adding KOH–methanol, usually at a concentration of 20% (w/v), in a volume similar to that of the ether extract, with the reaction being complete in 1 or 2 h, preferably in darkness and under an inert atmosphere of N_2 . The reaction is stopped by adding distilled water, and the phases are left to separate. The ether phase is washed repeatedly with distilled water to neutrality and finally washed with an aqueous solution of 2% (w/v) Na_2SO_4 . The extract is filtered through a solid bed of anhydrous Na_2SO_4 to completely remove water and taken to dryness in a rotary evaporator using temperatures below 30°C . The final result is a residue containing the carotenoid fraction, ready for subsequent analytical operations.

3.9.4 SEPARATION AND ISOLATION OF PIGMENTS

The method used to isolate carotenoid pigments depends mainly on their properties and their relative amounts in the food. After the operations of extraction and saponification (if the latter is necessary), the extract obtained will contain the carotenoid mixture to be separated. Normally, the extract will be composed of pigments of very different polarity (carotenes and xanthophylls), requiring an initial separation by phase distribution. Solvents of polarity similar to each pigment group will thus fractionate the extract prior to other separation techniques. Traditionally, the distribution and determination of the partition coefficients of carotenoids in systems of petroleum ether–(85 to 95% aqueous) methanol have been used to distinguish the fractions of carotenes and mono-, di-, and polyhydroxylated carotenoids, and the References contain extensive and detailed information (Davies, 1976; Foppen, 1971). The use of these techniques has been pushed into the background by current techniques of chromatographic separation.

Chromatographic techniques, in general, and that of column chromatography, in particular, were born out of the experiments of Tswett in 1906 to separate chlorophylls and carotenoids from leaf extracts (Tswett, 1906). The continuous development of different materials of adsorption, together with the appropriate use of solvents or mixtures of them, made this technique one of the most satisfactory isolation methods. Today, column chromatography (CC) and thin-layer chromatography (TLC) have become rather left behind by the enormous improvement in separations by high-performance liquid chromatography (HPLC), although their use is still necessary on many occasions. In all cases, the aforementioned general precautions required in the analysis of these compounds must be followed.

3.9.4.1 Column Chromatography

CC is used mainly in the separation of a mixture of carotenoids at a semipreparative or preparative scale, although subsequently TLC is required. The choice of a stationary phase in which the carotenoid mixture is adsorbed and separated depends on its selectivity and nonreactivity with the pigments and/or mobile phase. Table 3.6

TABLE 3.6
List of Adsorbents Used for Column
Chromatography of Carotenoids

Cellulose
Sucrose
Starch
CaCO ₃
Ca ₃ (PO) ₄
ZnCO ₃
Al ₂ O ₃
MgO
Ca(OH) ₂
CaO
Silica gel

shows some of the stationary phases most commonly used in CC of carotenoids. Pigment polarity partly determines the stationary phase to be used. Thus, the carotenes are separated better on columns of calcium hydroxide or alumina (deactivated to grade III). If the carotenoid fraction is of medium polarity, the use of calcium carbonate (CaCO₃) and magnesium oxide (MgO) is recommended. Highly polar xanthophylls require a stationary phase of weaker adsorption, such as cellulose.

The mobile phase chosen also depends on its polarity and that of the carotenoid mixture to be separated. Table 3.7 shows some of the solvents used as the mobile phase in CC of carotenoids. Some appropriate mixtures are diethyl ether, benzene or acetone in light petroleum ether, ethanol in diethyl ether, or ethyl acetate in benzene. The recovery of each pigment isolated is helped by using a solvent with a very low boiling point.

One parameter to consider after the choice of each phase is the length/diameter ratio of the column — the higher the ratio, the higher the efficiency of the chromatographic separation. Values of 8:1 to 20:1 give the best results. Generally, one third of the column is packed with adsorbent material, and the amount of sample is calculated with reference to the weight of packing (normally at a ratio of 1:100). A minimum amount of sample to be chromatographed is dissolved in a low-polarity solvent and added to the column. Once the mixture has been adsorbed by the packing, the chosen solvent is added for the separation at an outlet flow rate of 1 to 2 drops per second.

A precaution to bear in mind is that this technique does not allow uncolored compounds to be distinguished. Therefore, there is a considerable possibility that any carotenoid fraction isolated may be contaminated with other compounds of similar polarity but without color.

As a concrete example of CC separation of carotenoid pigments present in plants, this can be carried out by a prior separation on a silica column using methanol as the eluent (Lee, 1986; Lee et al., 1981). In this first separation, three fractions of different polarity (carotenes and mono- and polyhydroxylated xanthophylls) are

TABLE 3.7
Solvents Most Commonly Used for Column
Chromatography and Thin-Layer
Chromatography of Carotenoids

Light petroleum
n-Hexane
Cyclohexane
Carbon tetrachloride
Benzene
Toluene
Diethyl ether
Acetone
Ethyl acetate
Dichloromethane
tert-Butyl alcohol
n-Propanol
Ethanol
Methanol
Pyridine
Acetic acid in ethanol (1–10%, v/v)

Note: Solvents are listed from low to high polarity.

obtained. Each fraction can be rechromatographed to give a second separation. Thus, for instance, the carotene fraction of carrots, tomatoes, and maize is separated on a column of MgO-Hyflo Super Cel. Other adsorbents (CaCO₃, ZnCO₃, polyethylene, cellulose, etc.) have been used to separate the carotenoids of pepper, tomato, carrot, etc.

3.9.4.2 Thin-Layer Chromatography

The popularity of TLC lies in the versatility and efficiency of the separation achieved, enabling the subsequent quantification of each isolated pigment (Sherma, 2000; Stahl, 1967). Such characteristics, together with the ease of use, make this a technique still widely used, even in laboratories with more advanced analytical systems such as HPLC. In the particular case of the carotenoids, it can be considered a fundamental tool in identification. The use of TLC has been described in numerous publications, and it is common as a preliminary method of separation of carotenoid mixtures, for the purification of carotenoids previously separated by CC, and for the tentative identification of carotenoids depending on their chromatographic properties (especially the R_f value). The literature widely describes the properties of chromatographic separation and the R_f value for many pigments (Davies, 1976; Davies and Köst, 1988; Foppen, 1971).

TABLE 3.8
List of Adsorbents Used for Thin-Layer
Chromatography of Carotenoids

Cellulose
Sucrose
Mannitol
Kieselguhr
$\text{CaCO}_3\text{--MgO--Ca(OH)}_2$ (30:6:5)
$\text{Mg}_3(\text{PO}_4)_2$
$\text{ZnCO}_3\text{--Al}_2\text{O}_3$ (deactivated)
$\text{Mg}_2(\text{OH})_2\text{CO}_3$
MgO–kieselguhr (1:1)
MgO
MgOsilica gel (1:1)
Ca(OH)_2
$\text{Ca(OH)}_2\text{--silica gel}$ (6:1)
Silicic acid
Silica gel
Al_2O_3

Table 3.8 shows some adsorbents used to prepare the stationary phase in the chromatographic separation of carotenoids by TLC. The choice between them depends on the solvent or mixture of solvents to be used as the eluent phase. The adsorbent layer is placed on the glass plate (normally 20×20 cm) as a slurry, with a thickness that is variable but small (0.2 to 0.7 mm). The adsorbent is allowed to air dry and is activated in the oven at 110°C . The pigment extract is applied to the base of the plate, and the plate is put into a tank containing the eluent. Development is usually carried out upwards, and when complete, the band or bands of interest are selected, scraped off, eluted from the silica with either diethyl ether (in the case of polar carotenoids) or acetone or ethanol (if the polarity is medium), and filtered to remove the silica.

Among the general methods to separate carotenoids by TLC, that of Gross (1980) should be mentioned. This method uses a development on silica gel with acetone–light petroleum ether (30:70), giving a first separation into fractions of different polarity (carotenes and mono-, di-, and polyhydroxylated xanthophylls). Each group is then separated into the individual components by rechromatography on MgO–kieselguhr (1:1, w/w) with the same eluent mixture, but with higher proportion of acetone (4 to 30% volume), depending on the polarity of the pigment group to be separated.

Table 3.9 summarizes some TLC methods used for the analysis of carotenes and xanthophylls in various vegetables, fruits, and foods in general. Some commonly used conditions are the following. For the separation of chlorophylls and carotenoids in an olive extract, a mixture of light petroleum ether–acetone–diethylamine (10:4:1) can be used, with silica gel 60 GF₂₅₄ as the stationary phase (Mínguez-Mosquera and Garrido-Fernández, 1989). This method can also be used for the chromatographic separation of samples from green plants, in general, and for the separation

TABLE 3.9
Thin-Layer Chromatography Methods for Quantitative Determination of Carotenoids in Foods

Sample Type	Analyzed Carotenoids	Stationary Phase	Mobile Phase	Ref.
Green and red pepper fruits	Antheraxanthin, capsanthin, capsanthin-5,6-epoxide, capsorubin, β -carotene, β -carotene-5,6-epoxide, hydroxy- α -carotene, cryptocapsin, β -cryptoxanthin, lutein, neoxanthin, violaxanthin, zeaxanthin	Silica gel G	Benzene–ethyl acetate–methanol (75:20:5)	Davies et al., (1970)
Green and processed vegetables, ripe fruits (saponified extract)	β -Carotene, β -cryptoxanthin, lutein, neoxanthin, violaxanthin, neochrome, auroxanthin, zeaxanthin, capsanthin, capsorubin, cucurbitaxanthin A	Silica gel G	Light petroleum ether–acetone–diethylamine (10:4:1)	Mínguez-Mosquera and Garrido-Fernández (1989)
Ripe fruits (direct extract)	β -Carotene, β -cryptoxanthin, zeaxanthin, capsanthin, capsorubin	Silica gel G	Hexane–ethyl acetate–ethanol–acetone (95:3:2:2)	Mínguez-Mosquera et al., (1984)
Tomato fruits	Lycopene, prolycopene, violaxanthin, neoxanthin, <i>cis</i> -mutatoxanthin, lutein	Silica gel 60	Hexane–benzene–acetone–acetic acid (80:10:5:5)	Daood et al. (1987)
Tomato fruits	Lycopene, prolycopene, violaxanthin, neoxanthin, <i>cis</i> -mutatoxanthin, lutein	MgO–Hyflo Super Cel–cellulose (10:9:1)	Hexane–isopropanol–methanol (100:2:0.2)	Johjima and Ogura (1983)

of the pigments present in saponified extracts of various fruits. For the separation of the carotenoid mixture in a direct extract from fruits, the presence of esters of xanthophylls with fatty acids requires the use of mobile phases of lower polarity, such as the mixture hexane–ethyl acetate–ethanol–acetone (95:3:2:2) used for the analysis of esterified xanthophylls from the direct extract from fruits of red pepper (Mínguez-Mosquera et al., 1984). Carotenes from tomato are separated using the mixture hexane–isopropanol–methanol (100:2:0.2) on plates covered with MgO–Hyflo Super Cel–cellulose (10:9:1) (Johjima and Ogura, 1983).

Considerable improvements have been made in this separation technique. Adsorbents of high quality and more uniform particle size, including chemically bonded (C_{18} , C_8 , C_2) reversed phases, have been introduced, which allow an increased capacity of separation, resolution, and effectiveness, so that high-performance TLC (HPTLC) can be carried out.

3.9.4.3 High Performance Liquid Chromatography

HPLC methods have been used for the separation of carotenoids since the beginning of this technique in the 1970s. One of the first separations of carotenoids by HPLC was carried out by Stewart and Wheaton in 1971 from extracts of citrus fruits. Since then, important advances have been made in both the technique of HPLC and the development of chromatographic methods for the separation and detection of carotenoids.

In any chromatographic separation by HPLC, a series of factors must be added to the general precautions of management and analysis of carotenoid pigments. The solvents must be of high quality and, before their use, must be degassed to prevent the entrance of air into the chromatographic system and to minimize noise on the baseline. This is achieved using a prior sonication, filtration *in vacuo*, or a purging with helium. The mobile phase should always be filtered at 0.45 μm to remove any suspended particle. The use of a precolumn of the same packing as the main column is advisable to delay deterioration and the consequent decrease in separation efficiency of the analytical column. For the same reasons, the samples should be filtered at 0.45 μm or centrifuged at 12,000 rpm before being analyzed.

3.9.4.3.1 Instrumentation and Chromatographic Conditions

If a mixture of solvents is used as eluent, and whenever a gradient is employed, a binary, ternary, or quaternary pump is necessary. For isocratic separations, a simple pump is sufficient. Detection is usually carried out with a UV-visible spectrophotometric detector that can be of fixed or variable wavelength or a diode array. Detection is generally performed at around 450 nm, which is the region of maximum absorption for most carotenoids. The increasingly widespread use of diode array detectors allows recording of the complete absorption spectrum (range 350 to 550 nm), which, together with the chromatographic properties of the pigment, can provide very useful information for subsequent identification. Other detection systems such as a coulometric electrochemical detector have been successfully used for the analysis of carotenoids in human plasma (Ferruzzi et al., 1998).

Carotenoid separation can be carried out using both normal-phase HPLC (NP-HPLC) and reversed-phase (RP-HPLC). In the former, the stationary phase has a polar nature, with silica gel as the most commonly used packing, although it is also possible to use nitrile- or amino-type linked phases. The normal phase tolerates the presence of glycerides and any nonpolar matter, as it does not adsorb them strongly and can be removed later in a washing step. This type of stationary phase requires a mobile phase that is nonpolar or of low polarity. Thus, the most used solvent is hexane together with small amounts of other solvents of higher polarity (methanol, propanol, etc.); water is not recommended. RP-HPLC uses nonpolar stationary phases such as octyl silane (C_8), octadecyl silane (C_{18}), and polymers (polystyrene,

divinyl benzene, and polymethacrylate). The recent introduction of the C₃₀ stationary phase into RP-HPLC, enabling a significant increase in the interaction between analyte and solid phase compared with C₁₈, has meant a considerable advance in chromatographic resolving power in the separation of carotenoids (Sander et al., 2000; Van Breemen, 1996). The mobile phase comprises a mixture of polar solvents, generally methanol, acetonitrile, dichloromethane, and water, although nonaqueous systems are preferred for the chromatographic separation of carotenes. This type of chromatography requires the removal of nonpolar compounds (glycerides and fats in general) that occasionally accompany the sample, so a step of washing the column is required to ensure equal chromatographic conditions from one analysis to another.

In both types of chromatography (normal and reversed), the effectiveness of separation depends largely on particle size, which ranges between 3 and 10 μm and is commonly 5 μm . The quality of separation increases if the size is uniform throughout the packing. The column is usually of steel, typically 25 cm in length and 0.4 cm I.D., although other dimensions are also used, especially for preparative or semi-preparative HPLC applications.

Both NP-HPLC and RP-HPLC can either use isocratic elution systems, that is, of constant composition during the whole separation, or use an elution gradient that changes the composition of the eluent during analysis. Although there are many chromatographic conditions to achieve the separation of any mixture of carotenoid pigments, Khachick et al. (1992a) described three systems of general use for the separation of carotenoids present in foods (Table 3.10) using RP-HPLC, with a C₁₈ stationary phase and employing elution gradients or isocratic systems depending on the pigment fraction to be separated. Table 3.11 summarizes some methods used for HPLC analysis of carotenes and xanthophylls in foods.

Some of the methodologies used are outlined. For the separation of carotenes, interesting methods are described by Bushway and Wilson (1982), Bushway (1986), Lesellier et al. (1989), and Saleh and Tan (1991). O'Neil et al. (1991) evaluated the resolving capacity and quantification of (*Z/E*)- β -carotene isomers in four chromatographic columns with various solvent systems and found the Ca(OH)₂ column to be the best. Biacs et al. (1989) employed the isocratic mixture acetonitrile–2-propanol–water (39:57:4) to separate esterified carotenoids of paprika. Khachik et al. (1989) quantified the major carotenoids and their esters in apricot, peach, melon, and grape. Heinonen et al. (1989) used this last method to analyze the carotenoid content of 69 types of vegetable and fruit, both fresh and processed. Special attention has been given to the separation by HPLC of carotenoids with provitamin A activity.

Currently, the coupled combination of HPLC and mass spectrophotometric (MS) detectors enables the identification of carotenoid pigments without the need for the prior stages of isolation and purification and with the advantage of lower detection levels (Van Breemen, 1996).

3.9.4.4 Preparation of Standards

Excepting the carotenoids obtained at industrial scale and thus commercially available (β -carotene, canthaxanthin, astaxanthin, β -apo-8'-carotenal, β -apo-8'-carotenoic ethyl

TABLE 3.10
General Chromatographic Conditions for Carotenoid Pigments Separation in Foods as Proposed by Khachik et al. (1992a)

System	Column	Mobile Phase	Elution Gradient		Flow Rate
			Linear Gradient		
A	Octadecyl (C ₁₈)-silylated silica, 5 μm, 25 × 0.46 cm	Acetonitrile	0–10 min	40 min	0.7 ml/min
		Dichloromethane–hexane (1:1)	85%	45%	
		Methanol	5%	45%	
B	Octadecyl (C ₁₈)-silylated silica, 5 μm, 25 × 0.46 cm	Acetonitrile Dichloromethane Methanol	Isocratic		1 ml/min
			55%		
			23%		
			22%		
C	Octadecyl (C ₁₈)-silylated silica, 5 μm, 25 × 0.46 cm	Acetonitrile Methanol Dichloromethane–hexane (1:1)	Isocratic		0.7 ml/min
			85%		
			10%		
			5%		

ester, and citranaxanthin), carotenoid standards must be obtained either by total or partial synthesis or from natural sources in which their presence is confirmed using the extraction and separation techniques described previously. Table 3.2 (presence and distribution of the most common carotenoids in foods) can be used to choose the natural source from which carotenoid pigment standards can be obtained.

Carotenoids such as lutein, antheraxanthin, violaxanthin, and neoxanthin can be readily obtained from a saponified extract of pigments from spinach (*Spinacia oleracea*), alfalfa (*Medicago sativa*), or any other green plant. Lycopene, phytofluene, and ζ -carotene are isolated from a pigment extract of tomato. Zeaxanthin and β -cryptoxanthin are obtained from fruit extracts of peach (*Prunus armeniaca*) and papaya (*Carica papaya*) and other fruits. If the carotenoid pigments required are exclusively synthesized by and present in a plant genus, it is necessary to use their natural source. Such is the case of capsanthin and capsorubin present in the red pepper (*Capsicum annuum*), bixin and norbixin in the annatto seeds, cucurbitaxanthin A in pumpkin, lactucaxanthin in lettuce (*L. sativa*), or crocin in saffron anthers (*Crocus sativus*).

Some carotenoids are obtained in the laboratory from another related carotenoid by means of partial synthesis, which includes the reactions described in the identification of functional groups section. For example, auroxanthin and luteoxanthin are obtained from violaxanthin by acidification. Neochrome and mutatoxanthin are obtained from neoxanthin and antheraxanthin, respectively, using the same procedure (Khachik et al., 1986). The (Z)- β -carotene isomers are prepared by reflux heating

TABLE 3.11
High-Performance Liquid Chromatography Methods for Quantitative Determination of Carotenoids in Foods

Sample Type	Analyzed Carotenoids	Stationary Phase	Mobile Phase	Detection	Ref.
Green vegetables: potato leaves, cassava leaves, salad pea leaves, pumpkin leaves	β -Carotene	ODS-Hypersil, 3 μ m	Methanol–acetonitrile–chloroform–water (200:250:90:11)	Absorbance at 450 nm	Speek et al. (1986)
Sweet potato, carrot, squash, collard, cucumber, tomato, peach, apricot, nectarine, plum	9- <i>cis</i> -, 13- <i>cis</i> -, All- <i>trans</i> - β -Carotene	Ca(OH) ₂	Acetone–hexane (3:997)	Absorbance at 436 nm, and 340 nm for <i>cis</i> isomers	Chandler and Schwartz (1987)
Carrot, blueberry, potato	α -Carotene, β -carotene	Partisil 5 ODS	Acetonitrile–tetrahydrofuran–water (85:12.5:2.5)	Absorbance at 470 nm	Bushway and Wilson (1982)
Peas, carrot, sweet potato, kale, spinach, squash, apricot, peach	9- <i>cis</i> -, 13- <i>cis</i> -, All- <i>trans</i> - β -Carotene	Vydac 201 TP, 5 μ m	Methanol–chloroform (94:6)	Absorbance at 475 nm Uses Sudan 1 as internal standard	Quackenbush (1987)
Margarine	Carotenes and vitamin A	LiChrosorb Si-60, 5 μ m	Hexane–diethyl ether (92:8)	Absorbance at 453 nm	Thompson et al. (1980)
Red pepper	Capsorubin, violaxanthin, capsanthin-5,6-epoxide, capsanthin, antheraxanthin, luteoxanthin, mutatoxanthin, capsolutein, zeaxanthin, lutein, cryptoflavin, cryptocapsin, β -cryptoxanthin, β -carotene	Spherisorb silica, 5 μ m	A = light petroleum B = acetone 95% A to 75% A in 30 min and kept for 5 min Flow: 1.0 ml/min	Absorbance at 460 nm	Almela et al. (1990)

Red pepper, paprika, oleoresins	Capsorubin, violaxanthin, capsanthin-5,6-epoxide, capsanthin, 9- <i>cis</i> -capsanthin, 13- <i>cis</i> -capsanthin, antheraxanthin, mutatoxanthin, cucurbitaxanthin A (capsolutein), zeaxanthin, 9- <i>cis</i> -zeaxanthin, 13- <i>cis</i> -zeaxanthin, β -apo-8'-carotenal, cryptocapsin, β -cryptoxanthin, β -carotene, <i>cis</i> - β -carotene	Spherisorb ODS2, 5 μ m	A = acetone B = water 75% A for 5 min, to 95% A in 5 min, 95% A by 7 min, to 100% A in 3 min Flow: 1.5 ml/min	Absorbance at 450 nm Uses β -apo-8'-carotenal as internal standard	Mínguez-Mosquera and Hornero-Méndez (1993)
Olive and olive oil, and green vegetables (fresh and processed) in general	Neoxanthin, violaxanthin, lutein, antheraxanthin, mutatoxanthin, β -carotene, neochrome, auroxanthin, luteoxanthin, and chlorophylls	Spherisorb ODS2, 5 μ m	A = water-ion pair reagent-methanol (1:1:8) B = acetone-methanol (50:50) Ion pair reagent: tetrabutylammonium acetate 0.05 M — ammonium acetate 1 M 75% A to 75% B in 8 min, kept isocratic for 2 min, then to 90% B in 8 min (convex profile), to 100% B in 5 min (concave profile) Flow: 2 ml/min	Absorbance at 410, 430 and 450 nm	Mínguez-Mosquera et al. (1991b)
Oranje juice	α -Cryptoxanthin, β -cryptoxanthin, zeaxanthin, α -carotene, β -carotene, and <i>cis</i> isomers	Vydac 201 TP, 5 μ m	Methanol-chloroform (90:10) Flow: 1.0 ml/min	Absorbance at 475 nm	Quackenbush and Smallidge (1986)
Vegetables	Canthaxanthin, β -cryptoxanthin, α -carotene, γ -carotene, β -carotene, and <i>cis</i> isomers, lycopene	Vydac 218 TP, 5 μ m	Methanol-acetonitrile-tetrahydrofuran (52:40:8) Flow: 1.0 ml/min	Absorbance at 470 nm	Bushway (1985)

TABLE 3.11 (continued)
High-Performance Liquid Chromatography Methods for Quantitative Determination of Carotenoids in Foods

Sample Type	Analyzed Carotenoids	Stationary Phase	Mobile Phase	Detection	Ref.
Paprika	Capsorubin, violaxanthin, capsanthin, 9- <i>cis</i> -capsanthin, 13- <i>cis</i> -capsanthin, antheraxanthin, zeaxanthin, cryptocapsin, cryptoxanthin, β -carotene, and more than 30 esters	Zorbax ODS, 5 μ m	A = acetone–water (75:25) B = acetone–methanol (75:25) 100% A to 65% B in 10 min, to 80% B by 30 min, to 100% B by 60 min Flow: 1 ml/min	Absorbance at 460 nm	Fisher and Kocs (1987)
Vegetables	Neoxanthin, <i>cis</i> -neoxanthin, violaxanthin, neochrome, lutein 5,6-epoxide, lutein, <i>cis</i> -lutein, β -apo-8'-carotenal, β -carotene, 15- <i>cis</i> - β -carotene, luteoxanthin, auroxanthin, and some esters	Microsorb C ₁₈ , 5 μ m	A = Methanol–acetonitrile–dichloromethane–hexane (15:75:5:5) B = methanol–acetonitrile–dichloromethane–hexane (15:40:22.5:22.5) 100% A for 12 min, to 100% B in 15 min (linear) Flow: 0.5 ml/min	Absorbance at 450 nm	Khachik et al. (1986)
Fruit and vegetables	β -Carotene, α -carotene, β -cryptoxanthin	Partisil ODS, 5 μ m	Acetonitrile–tetrahydrofuran–water (85:12.5:2.5) Flow: 0.5 ml/min	Absorbance at 470 nm	Bureau and Bushway (1986)
Fruit and vegetables	Lutein, zeaxanthin, and rest of chloroplastic pigments	Shandon Hypersil, 5 μ m	Tetrahydrofuran–water (51:49) Flow: 1 ml/min	Absorbance at 450 nm	Juhler and Cox (1990)

at 200°C an acetone solution of all-(*E*)- β -carotene obtained from a pigment extract of carrot (Tsukida et al., 1982).

3.9.5 IDENTIFICATION

When the carotenoids have been isolated and purified, they are identified by their physicochemical and spectroscopic properties. Generally, carotenoid pigment identification has been carried out essentially from chromatographic and spectroscopic characteristics. According to Schiedt and Liaaen-Jensen (1995), for an identification to be considered acceptable today, it must include a series of minimum identification criteria: (1) the UV-visible absorption spectrum ($\lambda_{\max}$ and fine structure) must be in concordance with the proposed chromophore; (2) the chromatographic properties must be identical in two different systems, preferably TLC (R_f) and HPLC (t_R), and where possible, cochromatography with standards should be performed; and (3) the mass spectrum of pigments must be obtained in order to know at least the exact molecular weight. Modern assignment and complete elucidation of the structure of a carotenoid pigment includes the use of sophisticated spectroscopic techniques such as ^1H NMR (nuclear magnetic resonance), ^{13}C NMR, CD (circular dichroism), ORD (optical rotatory dispersion), and Raman spectroscopies, requiring the qualified use of complex instruments. Their application to carotenoid pigments has been the subject of specific monographs of essential reading for both newcomers and specialists (Buchecker and Noack, 1995; Englert, 1995; Koyama, 1995).

Routinely, the identification of a pigment begins with the study of the adsorption properties on chromatographic supports, with TLC and occasionally CC being the most used techniques. TLC gives preliminary information on color and, above all, mobility (R_f) of the pigment under the assay conditions (support and eluents). As the affinity of adsorption depends on many external factors, the R_f value is of little use except when cochromatography is performed with pure standards. If these are not available, they will have to be isolated in the laboratory from natural sources that contain them, as stated earlier in Section 3.9.4.4. The literature (Davies, 1976; Davies and Köst, 1988; Foppen, 1971) lists R_f values for many carotenoids under defined TLC conditions, and these can be useful in a first inspection when little is known about the nature of the pigment.

The chromatographic study must be complemented spectroscopically, with UV-visible spectroscopy being the most commonly used because of its availability and simplicity. The UV-visible absorption spectrum of a pure pigment is usually recorded in various solvents, and the values obtained for the absorption maxima and fine structure are compared with those listed in the References (Britton, 1995a; Davies, 1976; Foppen, 1971) and, if possible, with the spectrum of a pure standard recorded under the same conditions. In practice, it is normal to find differences of a few nanometers with respect to the $\lambda_{\max}$ values in the literature, due basically to instrument-related factors. Table 3.12 details the absorption maxima in different solvents for the carotenoid pigments commonly found in foods of plant and animal origins.

Currently, the use of HPLC coupled with detection systems such as spectrophotometric diode array detectors has meant a considerable advance in the identification

TABLE 3.12
Absorption Maxima (nm) for the Visible Light
Spectrum of Carotenoids in Different Solvents

Carotenoid	Absorption maxima			Solvent
	(λ_{max} , nm)			
Antheraxanthin	430	456	484	Chloroform
	422	445	472	Light petroleum
	422	444	472	Ethanol
Astaxanthin		468		Light petroleum
		478		Ethanol
		480		Acetone
Auroxanthin	378	400	424	Light petroleum
	380	400	422	Ethanol
Bixin	432	456	490	Light petroleum
	433	470	502	Chloroform
Canthaxanthin		466		Light petroleum
		474		Ethanol
		482		Chloroform
Capsanthin	450	475	505	Light petroleum
	460	483	518	Benzene
Capsorubin	445	479	510	Light petroleum
	460	489	524	Benzene
α -Carotene	422	444	473	Light petroleum
	422	444	472	Ethanol
	424	448	476	Acetone
β -Carotene	433	457	484	Chloroform
	425	449	476	Light petroleum
		450	476	Ethanol
	429	452	478	Acetone
	435	461	485	Chloroform
δ -Carotene	431	456	489	Light petroleum
	440	470	503	Chloroform
γ -Carotene	437	462	494	Light petroleum
	439	461	491	Acetone
ϵ -Carotene	416	440	470	Light petroleum
	417	440	470	Ethanol
ζ -Carotene	378	400	425	Light petroleum
	377	399	425	Ethanol
Crocin	400	422	450	Light petroleum
	413	435	462	Chloroform
α -Cryptoxanthin	421	445	475	Light petroleum
	434	456	485	Chloroform
β -Cryptoxanthin	425	449	476	Light petroleum
	428	450	478	Ethanol
Cryptoxanthin-5,6-epoxide	418	443	470	Light petroleum
	424	447	476	Ethanol
Cucurbitaxanthin A	423	445	473	Ethanol

TABLE 3.12 (continued)
Absorption Maxima (nm) for the Visible Light
Spectrum of Carotenoids in Different Solvents

Carotenoid	Absorption maxima			Solvent
	(λ_{max} , nm)			
Lactucaxanthin	434	458	486	Benzene
		438	468	Light petroleum
	419	440	470	Ethanol
Lutein	421	445	474	Light petroleum
	422	445	474	Ethanol
	435	458	485	Chloroform
Luteoxanthin	402	426	448	Light petroleum
	400	420	446	Ethanol
Lycopene	444	470	502	Light petroleum
	446	472	503	Ethanol
	448	474	505	Acetone
	458	484	518	Chloroform
Mutatoxanthin	402	424	448	Light petroleum
	410	434	460	Chloroform
Neochrome	399	418	446	Light petroleum
	402	426	454	Chloroform
Neoxanthin	416	438	467	Light petroleum
	415	439	467	Ethanol
Neurosporene	423	448	476	Chloroform
	414	439	467	Light petroleum
	416	440	470	Hexane
	416	440	469	Ethanol
	424	451	480	Chloroform
Norbixin	442	474	509	Chloroform
Phytoene	276	286	297	Light petroleum
Phytofluene	331	348	367	Light petroleum
Violaxanthin	416	440	465	Light petroleum
	419	440	470	Ethanol
	426	449	478	Chloroform
Zeaxanthin	424	449	476	Light petroleum
	428	450	478	Ethanol
	430	452	479	Acetone
	433	462	493	Chloroform

of carotenoid pigments, enabling chromatographic and spectroscopic information to be obtained at the same time. Furthermore, the greater resolving power of HPLC and the diversity of chromatographic supports have enabled the tackling of problems difficult to solve by classic techniques, such as the separation of optic or chiral isomers.

In addition, the identification of carotenoid pigments includes the characterization of the functional groups, which can occasionally be inferred from the UV-visible

spectrum. IR spectroscopy, although not very applicable to the identification of carotenoid pigments, is especially selective in determining the presence of particular functional groups such as acetylenic, allenic, hydroxyl, and carbonile. In contrast, MS of carotenoid pigments has become an essential technique because of the valuable information it gives about functional groups and substituents in general. MS of carotenoid pigments is normally performed using electron impact (EI) as method of ionization. This is applied directly to the sample (direct probe) subjected to a temperature of around 200°C. The presence of functional groups in a carotenoid will be shown by a characteristic fragmentation: for instance, hydroxyl groups give rise to the loss of water (18 mass units), especially intense in the case of hydroxyl groups at allyl positions. The presence of 5,6- and 5,8-epoxide groups gives rise to the abundant appearance of fragments of 80, 165, and 205 mass units. The presence of β and ϵ end rings can also be determined from the appearance of characteristic fragments. In the case of the central polyene chain, characteristic fragments of it are usually present in the mass spectra of most carotenoids, very frequently in fragments of 92 mass units (loss of toluene) and 106 mass units (loss of *m*-xylene). Most carotenoids subjected to ionization by EI give rise to an abundant molecular ion that enables knowledge of the molecular mass. The application of MS has been discussed in detail in various articles and reviews (Enzell and Back, 1995; Vetter et al., 1971).

The presence of the most characteristic and common functional groups can also be established using specific chemical reactions such as those described below.

3.9.5.1 Test for 5,6-Epoxy Groups

The test is based on chromophore modification resulting from the transformation of a 5,6-epoxide group to 5,8-epoxide in an acid medium. This structural transformation means the loss of the conjugated double bond at positions 7 and 8 of the central polyene, causing a hypsochromic displacement of the absorption spectrum of 15 to 20 nm. Chromatographically, the 5,8-epoxide derivatives are more polar, so chromatographic development by TLC using silica gel as support reveals bands with lower R_f values.

In practice, the test can be performed *in situ* on the chromatographic plate, subjecting the pigments to HCl fumes after the chromatographic separation. The appearance of a characteristic blue color identifies the presence of 5,6-epoxide groups: diepoxides give a deep blue color and monoepoxides give a greenish-blue color. The test is more useful when carried out in the spectrophotometric cuvette, recording the electron absorption spectrum before and after adding a few drops of dilute HCl to the pigment dissolved in ethanol. A hypsochromic displacement of 15 to 20 nm indicates the presence of a single 5,6-epoxide group, while a displacement of 35 to 40 nm indicates the presence of two 5,6-epoxide groups (Figure 3.8).

3.9.5.2 Test for Reduction of Carbonyl Groups

LiAlH_4 or NaBH_4 are usually used as reducing agents of carbonyl groups, forming the corresponding alcohols. The higher reducing power of LiAlH_4 allows the reduction not

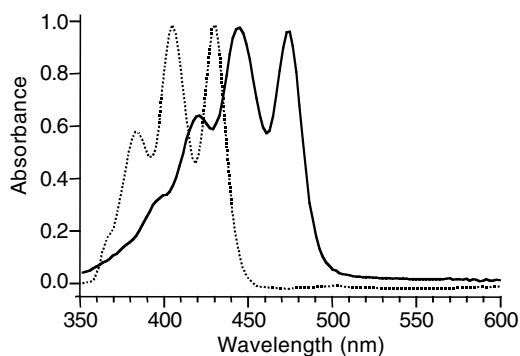


FIGURE 3.8 Spectroscopic test for 5,6-epoxide groups. Light absorption spectra of violaxanthin (—) and auroxanthin (.....) in ethanol.

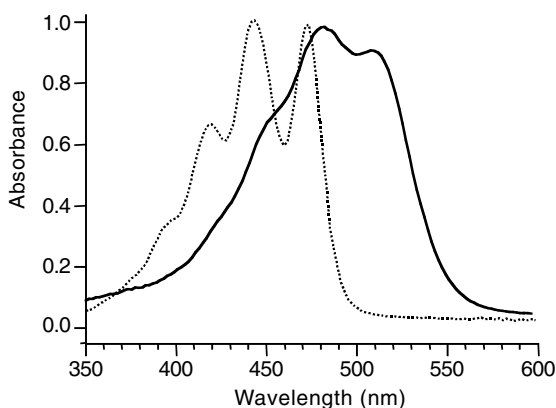


FIGURE 3.9 Spectroscopic test for carbonyl groups. Light absorption spectra of capsorubin (—) and the reduced product capsorubol (.....) in ethanol.

only of aldehydes and ketones, but also of acids, esters, and other polar functional groups.

The test is made starting from a solution of the pigment in ethanol. Some crystals of NaBH_4 are added, and the reaction is kept in the refrigerator and darkness for 3 h. The pigment is transferred to diethyl ether, and the electron absorption spectrum is recorded. In the case of carbonyl groups and those conjugated with the central polyene, reduction is shown by a hypsochromic displacement of the absorption maxima in the electron adsorption spectrum and a considerable increase in the fine structure (Figure 3.9). Larger hypsochromic displacements are characteristic of carbonyl groups situated on the polyene chain and whose reduction largely cleaves the conjugation. At the chromatographic level, the reduction of carbonyl groups results in greater polarity of the reaction product.

3.9.5.3 Test for Acetylation of Hydroxyl Groups

The test is based on the conversion of alcohols to esters by reaction with carboxylic acids or acid chlorides.

The test is made starting from a solution of pigment in pyridine (2 ml), to which acetic anhydride (0.2 ml) is added. The reaction is kept in darkness for 12 h and stopped by adding water. The pigments are transferred to petroleum ether. Chromatographic analysis by TLC or HPLC of the reaction mixture shows the number of hydroxyl groups in the carotenoid. The presence of a single hydroxyl group gives rise to a single acetylated derivative, and the presence of two results in one diacetylated derivative and one or two monoacetylated derivatives, depending on whether the position of hydroxyl groups are symmetric or not.

3.9.5.4 Test for Allyl Hydroxyl Groups

This functional group is identified by causing a dehydration, which introduces an additional double bond into the chromophore and originates a bathochromic displacement of 10 to 16 nm. The test is carried out subjecting the pigment, dissolved in chloroform, to the action of dilute HCl.

3.9.6 QUANTIFICATION

3.9.6.1 Quantitative Determination by UV-Visible Spectrophotometry

As discussed previously, the existence of an extensive system of conjugated double bonds making up the structural chromophore of the carotenoids is responsible for the characteristic absorption of light in the near UV range and, above all, in the visible range. This property has long been used for the routine quantification of carotenoids in solution, according to the law of Beer–Lambert:

$$A = A_{1\text{ cm}}^{1\%} \times L \times C$$

In practice, the main problem when applying this law is to know the exact value of the specific absorption coefficient ($A_{1\text{ cm}}^{1\%}$) or molar extinction coefficient (ϵ_{mol}) for a given carotenoid. The estimation of these values is not simple, and requires isolating and completely purifying the pigment, exactly weighing 1 to 2 mg, and making the spectrophotometric recording in a 1% (w/v) solution. By definition, the specific absorption coefficient ($A_{1\text{ cm}}^{1\%}$) represents the theoretical absorbance of the solution of 1 g of pigment in 100 ml of solvent (C, concentration) measured in a cuvette of 1-cm light path (L).

Values of absorption coefficients for most carotenoids commonly found in foods of plant and animal origins have been published in the various monographs on carotenoids (Britton, 1995a; Davies, 1976; Foppen, 1971). Nevertheless, caution is advisable when using this type of reference information: the values of $A_{1\text{ cm}}^{1\%}$ or ϵ_{mol} should be contrasted with other sources, as it has been demonstrated that there are discrepancies due mainly to the fact that in the beginnings of working with carotenoids, isolation and purification of pigments was not totally satisfactory, and the presence of impurities (mainly uncolored ones) caused errors in the estimation of

the coefficients. Currently, the need to revise the data published in the References is under discussion.

When the absorption coefficient is not known, an average value for $A_{1\text{ cm}}^{1\%}$ of 2500 is commonly taken. This is also applied to calculate the carotenoid content in an extract with a mixture of pigments. Another approach that can be used is to remember that carotenoids with the same chromophore must in theory have the same ϵ_{mol} value. Thus, if we know this value for a carotenoid, we can apply it to another having the same chromophore.

As a rule, the carotenoid (or carotenoid mixture in the case of an extract) to be quantified is dissolved in a known volume of an appropriate solvent (normally hexane, light petroleum ether, acetone, or ethanol). As in all spectrophotometric measurements, the spectrophotometric reading should be between 0.3 and 0.7 units to ensure linearity of the measurement and to minimize instrument errors. One of the most common sources of error is the insufficient or poor solution of the pigments, mainly when they are in crystalline form; this may require the use of a small amount of a strong solvent such as dichloromethane, chloroform, or tetrahydrofuran. When the carotenoid has been dissolved, a portion is placed in the quartz spectrophotometric cuvette (1-cm light path), and the value of absorbance (A) at the appropriate wavelength is recorded. The measurement is usually performed at the wavelength of the greatest absorption maximum (normally the centra, II). Today, with the spreading use of diode array spectrophotometers, it is possible to obtain instantly the absorption spectrum over the whole visible range of wavelength with a precision of 1 to 2 nm, enormously facilitating the localization of the absorption maxima.

The amount X (milligrams) of a carotenoid present in a volume V (milliliters) of solution can be calculated from the following equation:

$$X = (A \times V \times 1000) / (A_{1\text{ cm}}^{1\%} \times 100)$$

If the value of ϵ_{mol} is known, it is useful to apply the mathematical relationship with $A_{1\text{ cm}}^{1\%}$:

$$\epsilon_{\text{mol}} = (A_{1\text{ cm}}^{1\%} \times \text{molecular weight}) / 10$$

Table 3.13 shows the values of $A_{1\text{ cm}}^{1\%}$ for some of the most common carotenes and xanthophylls.

3.9.6.2 Quantitative Determination by Separation by TLC and UV-Visible Spectrophotometry

After chromatographic separation of the carotenoids present in a sample, the bands corresponding to individual pigments can be recovered from the chromatographic support by scraping each one off, followed by elution of the pigment with diethyl ether or acetone. The chromatographic adsorbent is removed by filtration, and the pigment is taken to known volume, normally 5 to 25 ml. Next, the electronic absorption spectrum is obtained and quantified spectrophotometrically as described previously. In order to know the carotenoid content in a food sample, the analyst

TABLE 3.13
Specific Absorption Coefficients ($A_{1\text{ cm}}^{1\%}$) Used
for Quantitative Spectrophotometric
Determination of Carotenoids

Carotenoid	$A_{1\text{ cm}}^{1\%}$	λ (nm) for Measurement	Solvent
Antheraxanthin	2350	446	Ethanol
Astaxanthin	2100	470	Hexane
Bixin	4200	456	Light petroleum
Canthaxanthin	2200	466	Light petroleum
Capsanthin	2072	483	Benzene
Capsorubin	2200	489	Benzene
α -Carotene	2800	444	Light petroleum
β -Carotene	2592	449	Light petroleum
δ -Carotene	3290	456	Light petroleum
γ -Carotene	3100	462	Light petroleum
ϵ -Carotene	3120	440	Light petroleum
ζ -Carotene	2555	400	Hexane
Crocin	4320	450	Light petroleum
α -Cryptoxanthin	2636	445	Light petroleum
β -Cryptoxanthin	2386	449	Light petroleum
Lutein	2550	445	Ethanol
Lycopene	3450	470	Light petroleum
Neoxanthin	2243	439	Ethanol
Neurosporene	2918	440	Hexane
Phytoene	1250	286	Light petroleum
Phytofluene	1350	348	Light petroleum
Violaxanthin	2250	440	Ethanol
Zeaxanthin	2348	449	Light petroleum

needs to know exactly the weight of the sample extracted [W (g)], the final volume of the extract [V_e (ml)], the volume of extract chromatographed [V_{cr} (ml)], and the final volume of elution of the pigment chromatographed [V_f (ml)]. The following equation gives the content (in milligrams per kilogram) for a determined pigment:

$$C = \frac{A \times V_e \times V_f}{A_{1\text{ cm}}^{1\%} \times W \times V_{cr}}$$

where

C = concentration (mg/kg)

V_e = initial volume of pigment extract (ml)

V_f = final volume of pigment eluent (ml)

W = weight of sample (g)

V_{cr} = volume chromatographed (ml)

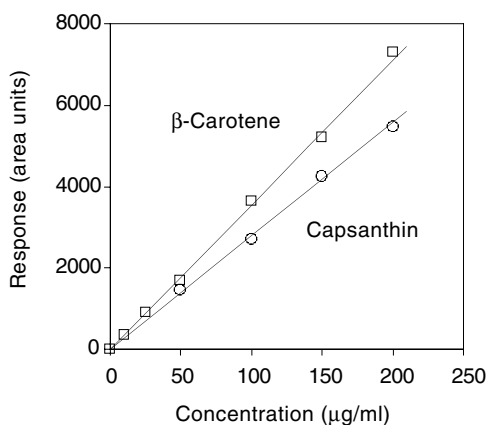


FIGURE 3.10 Calibration plots for UV-visible spectrophotometric determination of capsanthin and β -carotene after HPLC separation using 450 nm as the detection wavelength.

3.9.6.3 Quantitative Determination by HPLC and UV-Visible Spectrophotometric Detection

The now-routine chromatographic technique of HPLC has been in use since the 1970s for the quantification of carotenoid pigments. It is particularly sensitive when coupled with detection methods based on UV-visible spectrophotometry. When the pigments have been separated, they are quantified by detection at wavelengths as close as possible to $\lambda_{\max}$ of each pigment and the recording of the corresponding chromatogram. The ratio between absorbance and the amount (concentration) of pigment defined by the law of Beer-Lambert is calculated to relate the chromatographic peak area and amount of pigment. A detection wavelength of 450 nm is used for the routine detection of carotenoid pigments when using a fixed-wavelength detector. In the case of multiple-wavelength detectors, several chromatograms can be recorded at different detection wavelengths, choosing those that coincide with $\lambda_{\max}$ of the major pigments. The use of variable-wavelength detectors, and the modern diode array ones, helps to acquire chromatograms at $\lambda_{\max}$ of each pigment, especially when using automated systems that include computerized data treatment and storage.

The determination of each pigment concentration in a given sample requires a calibration to be carried out. Often, many workers have opted for a single calibration using β -carotene or another commercially available carotenoid as the standard. This simplification is bad practice and, in many cases, has resulted in serious quantitative errors. For instance, quantitative determination of capsanthin using the calibration plot obtained for β -carotene, using 450 nm as the detection wavelength, causes an underestimation (more than 30%) of capsanthin, whose $\lambda_{\max}$ is around 475 nm (Figure 3.10). Thus, although more complex and tedious, the best option is to perform an individual calibration for each pigment present in the sample, requiring a further extraction, separation, isolation, and purification. The stock solution of each pigment is quantified spectrophotometrically as described earlier, so it will be

essential to know $A_{1\text{ cm}}^{1\%}$ exactly. The calibration plot is obtained from the representation of concentration or amount of pigment vs. the peak area measured after injecting aliquots (normally 10 to 20 μl) of solutions of increasing concentration.

In order to avoid quantification errors caused by the multiple manipulations of the sample during the various steps of extraction and preparation, the use of an internal standard (IS) in combination with the external calibration is advisable. The internal standard must be chosen carefully, as it has to meet a series of minimum requirements. It must be a carotenoid pigment not present in the sample to be analyzed; it must be chromatographically separable from the others under the analytical conditions used; it must have a λ_{max} of absorption as close as possible to the λ of detection employed; and it must be as stable as possible. Various internal standards have been proposed: β -apo-8'-carotenal and canthaxanthin are commonly used in the analysis of vegetable foods (Mínguez-Mosquera and Hornero-Méndez, 1993). The use of artificial colorants, such as Congo red and Sudan 1, and of synthetic carotenoids not present in natural samples, such as C45- β -carotene, has also been proposed (Philip and Chen, 1988; Stewart and Wheaton, 1971).

As a practical example and reference, we now describe in detail the HPLC method for separation and quantification of carotenoid pigments in foods of different origin: (1) green vegetables, (2) ripe fruits, (3) processed vegetables, and (4) animal products.

3.9.6.3.1 Analysis of Carotenoid Pigments in Green Vegetables

Not every chromatographic method can be used to analyze carotenoid pigments in green plants, since not all of them separate the chlorophylls present in the resulting extract. In such case, saponification is normally used to remove them. The joint analysis of carotenoids and chlorophylls is extremely complex; when the latter are the object of study, their chromatographic analysis will have to be from a direct extract (see Chapter 4). Regarding the carotenoids, their analysis from a direct extract in green plants has certain advantages, among which stands out the lack of need for saponification which, if not carried out carefully, can give rise to quantitative loss and the appearance of artifacts. Described next is the method used routinely in the authors' laboratory for the analysis of chlorophylls and carotenoids in green plant tissues (Mínguez-Mosquera et al., 1991b). This method was initially proposed for the study of chloroplast pigments in olives, but has subsequently been used with success for the analysis of these pigments in many green plants.

Separation is performed on an RP column (Spherisorb ODS2, 5 μm , 25 $\times$ 0.46 cm). The eluents used are (A) water-ion pair reagent-methanol (1:1:8) and (B) acetone-methanol (1:1). The ion pair reagent, a solution of tetrabutylammonium acetate (0.05 *M*) and ammonium acetate (1 *M*) in water, is added to improve the separation of chlorophyll derivatives (Mantoura and Llewellyn, 1983). The pigment extract is prepared from 10 g of fresh sample and taken to a final volume with acetone (2 to 5 ml). Injection (20 μl) into the chromatograph is done after centrifuging the sample at 12000 rpm. Elution is carried out at a flow rate of 2 ml/min with the following gradient system: from an initial 75% A linearly to 25% A in 8 min, the composition is kept constant for 2 min, and then to 10% A in 8 min following a convex profile (curve 4 in the gradient profile of the Waters 600 E quaternary

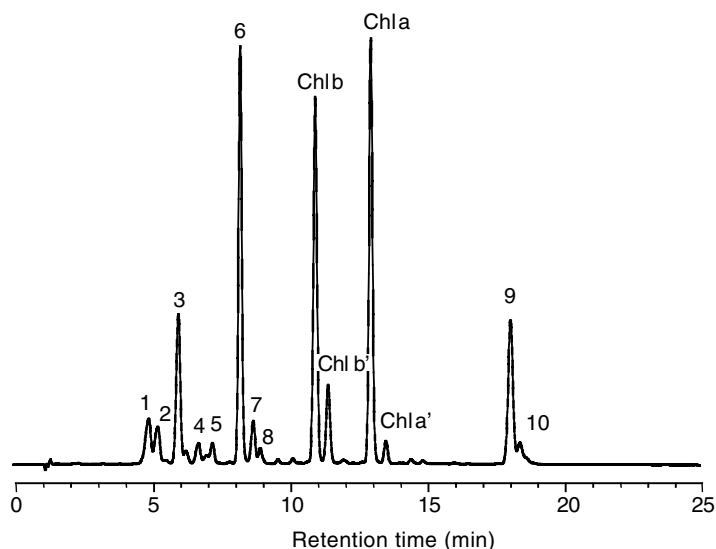


FIGURE 3.11 Carotenoid HPLC profile of a pigment extract from a fresh green vegetable (spinach). Peaks: 1, neoxanthin; 2, neoxanthin isomer; 3, violaxanthin; 4, luteoxanthin; 5, antheraxanthin; 6, lutein; 7, 9-*cis*-lutein; 8, 13-*cis*-lutein; 9, β -carotene; 10, *cis* β -carotene isomers. The sample also included chlorophyll pigments: chlorophyll *a* (Chl *a* and Chl *a'*) and chlorophyll *b* (Chl *b* and Chl *b'*). Mobile phase: A, water–ion pair reagent (tetrabutylammonium acetate 0.05 *M*–ammonium acetate 1 *M*)–methanol (1:1:8); B, acetone–methanol (50:50); flow rate, 2 ml/min; detection at 450 nm (Mínguez-Mosquera et al., 1991b).

pump), then to 100% B in 5 min via a concave profile (curve 10). The column is conditioned between successive injections, allowing 7 to 10 min to re-establish the initial conditions. Detection is performed with a diode array spectrophotometric detector (Waters 996) at two wavelengths, 430 and 450 nm, to enable joint monitoring of chlorophylls and carotenoids. Figure 3.11 shows a typical chromatogram for the separation of chloroplast pigments included in an extract of a green plant tissue (e.g., fresh spinach).

3.9.6.3.2 Analysis of Carotenoid Pigments in Ripe Fruits

The analysis of carotenoid pigments in fruits is more complex than in green plants. The number of possible carotenoids is higher, and there is no typical composition profile because of the wide structural diversity of carotenes and (especially) xanthophylls present. Moreover, fruit ripening generally involves three simultaneous processes: the disappearance of chlorophylls, the massive biosynthesis of carotenoid pigments, and esterification of the xanthophylls with fatty acids. This makes chromatographic separation and, in many cases, pigment identification, extremely complicated, with saponification being necessary in the preparation of the extract for its analysis by HPLC. We now describe the method used routinely by the authors for the analysis and quantification of carotenoids in ripe fruits of red pepper and its industrial derivatives, paprika and oleoresins (Mínguez-Mosquera and Hornero-Méndez, 1993). This method has been used successfully for the analysis of carotenoids in other carotenogenic fruits such as

tomato, persimmon, carrot, melon, and pumpkin and for quality control and authentication of concentrates and juices.

The chromatographic separation is carried out on an RP column (Spherisorb ODS2, 5 μ m, 25 $\times$ 0.46 cm). The eluents used are (A) acetone and (B) water. The pigment extract is prepared from some 10 g of fresh sample taken to volume with acetone (10 to 25 ml). Injection (10 μ l) into the chromatograph is done after centrifuging the sample at 12000 rpm. Elution is performed at a flow rate of 1.5 ml/min using the following gradient system: from an initial 75% A, kept constant for 5 min; linearly to 95% A in 5 min; where the composition is kept constant for 7 min; and finally to 100% A in 3 min. The column is conditioned between successive injections, allowing 5 min to reestablish the initial conditions. Detection is performed with a spectrophotometric detector at 450 nm. Quantification is carried out using β -apo-8'-carotenal as the internal standard, which is added in known amount at the beginning of extraction. Figure 3.12 shows the chromatograms for a direct extract and a saponified one of carotenoid pigments of ripe fruits of red pepper.

3.9.6.3.3 Analysis of Carotenoid Pigments in Processed Vegetables

During the different stages in the processing of vegetables the structure and stability of the chloroplast pigments may be affected, resulting in changes in the qualitative and quantitative composition of the final product. The transformations will depend on the type of process (fermentation, freezing, blanching, dehydration, etc.) and on the severity of the conditions applied. In the case of the carotenoid pigments, the most usual transformations are those involving fermentation, treatment with acids, or heating. In the case of fermentation and acid treatment, the most frequent change is the appearance of 5,8-epoxide derivatives from carotenoids having 5,6-epoxide groups, which are generally present in all green plants. In the case of heat processes, the main consequence is the formation of *cis* isomers and quantitative losses, directly affecting the provitamin A value.

For the analysis of chlorophylls and carotenoids, and their derivatives, in processed green plants, the previously described method (Mínguez-Mosquera et al., 1991b) can also be used. Figure 3.13 shows the chromatogram for the separation of chloroplast pigments of a processed green plant, green table olives, whose processing includes a fermentation step.

3.9.6.3.4 Analysis of Carotenoid Pigments in Foods of Animal Origin

Foods of animal origin contain very variable amounts of carotenoids, always incorporated via the diet because of the incapacity to synthesize them. Nevertheless, some animals are able to transform certain carotenoids, so together with the carotenoids coming from ingested foods we find their metabolites. Although the carotenoid profile found in a food of animal origin is determined by the type of diet, it is the bioavailability of each carotenoid that determines its presence and levels. Foods of animal origin that contain carotenoids include fish (salmon, trout, etc.), crustaceans (lobster, prawn, etc.), butter and other animal fats, milk, and birds' eggs. The carotenoids most commonly found in animal products are lutein, zeaxanthin, β -cryptoxanthin, β -carotene, α -carotene, canthaxanthin, lycopene, astaxanthin, and different apocarotenoids coming from the metabolization of these.

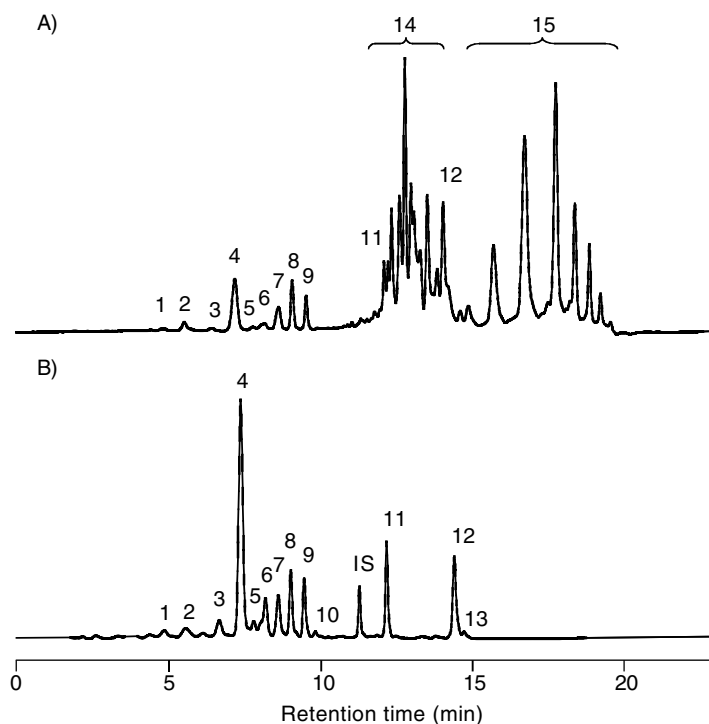


FIGURE 3.12 Carotenoid HPLC profile of a pigment extract from a ripe fruit (red pepper, *Capsicum annuum*): A, direct extract (nonsaponified); B, saponified extract. Peaks: 1, capsorubin; 2, violaxanthin; 3, capsanthin-5,6-epoxide; 4, capsanthin; 5, 13-*cis*-capsanthin; 6, 15-*cis*-capsanthin; 7, antheraxanthin; 8, cucurbitaxanthin a; 9, zeaxanthin; 10, 9-*cis* and 13-*cis*-zeaxanthin; 11, β -cryptoxanthin; 12, β -carotene; 13, 9-*cis* and 13-*cis*- β -carotene; 14, partially esterified xanthophylls; 15, totally esterified xanthophylls; IS the internal standard. Mobile phase: A, acetone; B, water, gradient elution; flow rate, 1.5 ml/min; detection at 450 nm (Mínguez-Mosquera and Hornero-Méndez, 1993).

For the analysis of carotenoids in animal products, saponification is normally advisable to remove the fats, although this should be avoided in the case of astaxanthin because of its lability to alkalis. The HPLC chromatographic methods for this type of analysis are varied, and although those described above can be used in many cases, there are specific methods in the References. Once the pigment extract has been prepared in each particular case, any HPLC chromatographic method for the analysis of carotenoids in blood plasma can be used. The method employed by Khachik's group is ideal, with the separation of a wide range of carotenoids and their metabolites (Khachik et al., 1992b). The chromatographic separation is performed on an RP column (Rainin Microsorb 5 μ m C₁₈, 25 $\times$ 0.46 cm). The eluents used are (A) acetonitrile, (B) dichloromethane–hexane (1:1), and (C) methanol. Elution is carried out at a flow rate of 0.7 ml/min with the following gradient system: from an initial 85% A to 5% B, kept constant for 10 min, then linearly to 45% A to 45% B in 30 min. Detection is performed simultaneously at 470, 445, 400, 350, and

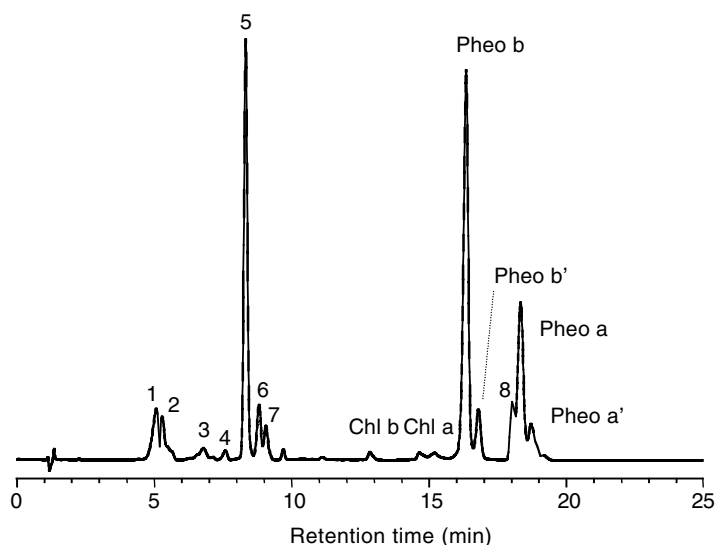


FIGURE 3.13 Carotenoid HPLC profile of a pigment extract from a processed green vegetable (olive). Peaks: 1, neochrome; 2, neochrome isomer; 3, luteoxanthin; 4, auroxanthin; 5, lutein; 6, 9-*cis*-lutein; 7, 13-*cis*-lutein; 8, β -carotene. Sample also included chlorophyll-derived pigments: chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), pheophythin *a* (Pheo *a* and Pheo *a'*), and pheophythin *b* (Pheo *b* and Pheo *b'*). Mobile phase: A, water–ion pair reagent (tetrabutylammonium acetate 0.05 *M* – ammonium acetate 1 *M*)–methanol (1:1:8), B, acetone–methanol (50:50); flow rate, 2 ml/min; detection at 450 nm (Mínguez-Mosquera et al., 1991b).

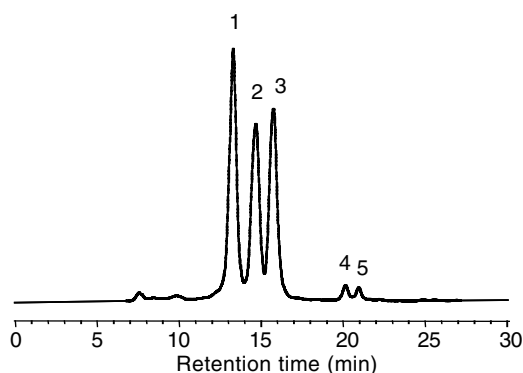


FIGURE 3.14 Carotenoid HPLC profile of a pigment extract from animal product (egg yolk). Peaks: 1, canthaxanthin; 2, lutein; 3, zeaxanthin; 4, α -carotene; 5, β -carotene. Mobile phase: A, acetonitrile; B, dichloromethane–hexane (1:1); C, methanol; flow rate, 0.7 ml/min; detection at 470 nm (Khachik et al., 1992b).

290 nm using a spectrophotometric detector. Quantification is done using β -apo-8'-carotene-3,8'-diol as the internal standard. Figure 3.14 shows a chromatogram for an extract of carotenoid pigments from egg yolk using the methodology described.

3.9.6.4 Determination of Provitamin A Value

The provitamin A value of a food is calculated using the equivalence proposed by the FAO/WHO (1967), such that one equivalent of retinol corresponds to 6 μ g of β -carotene. One equivalent of retinol is also equal to 10 international units (IU) of provitamin A activity calculated from β -carotene and 20 IU of activity from the rest of the carotenoids with provitamin A activity.

1 Eq. retinol = 6 μ g of β -carotene = 10 IU provitamin A activity

1 Eq. retinol = 12 μ g of the rest of carotenoids with provitamin A activity = 20 IU provitamin A activity

Apart from β -carotene, with maximum provitamin A activity, the other pigments meeting the above-discussed requirements for possession of provitamin A activity have to be considered. Of these, the most commonly found in foods are α -carotene, β -cryptoxanthin, α -cryptoxanthin, γ -carotene, mutatochrome, *cis* isomers of β -carotene, β -zeacarotene, and β -apo-8'-carotenal, which is added as colorant to many foods, although it can be found naturally in oranges and other citrus fruits. As these carotenoids present, at most, 50% of the activity of β -carotene (see Table 3.3), the provitamin A content of a food can be calculated from the following expression:

$$\text{Eq. retinol} = [\mu\text{g of } \beta\text{-carotene}/6 + \mu\text{g of the rest of the active carotenoids}/12]$$

REFERENCES

- Almela, L., Lopez-Roca, J.M., Candela, M.E., and Alcazar, M.D., Separation and determination of individual carotenoids in a capsicum cultivar by normal-phase high-performance liquid-chromatography, *J. Chromatogr.*, 502, 95–106, 1990.
- Armstrong, G.A., Eubacteria show their true colors — Genetics of carotenoid pigment biosynthesis from microbes to plants, *J. Bacteriol.*, 176, 4795–4802, 1994.
- Bartley, G.E. and Scolnik, P.A., Molecular biology of carotenoid biosynthesis in plants, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 45, 287–301, 1994.
- Bartley, G.E. and Scolnik, P.A., Plant carotenoids: pigments for photoprotection, visual attraction, and human health, *Plant Cell*, 7, 1027–1038, 1995.
- Bauernfeind, J.C., Carotenoid vitamin A precursors and analogs in foods and feeds, *J. Agric. Food Chem.*, 20, 456–473, 1972.
- Bendich, A., Carotenoids and the immune-response, *J. Nutr.*, 119, 112–115, 1989.
- Bendich, A. and Olson, J.A., Biological actions of carotenoids, *FASEB J.*, 3, 1927–1932, 1989.
- Biacs, P.A., Daoud, H.G., Pavis, A., and Hajdu, F., Studies on the carotenoid pigments of paprika (*Capsicum annum* L. var Sz-20), *J. Agric. Food Chem.*, 37, 350–353, 1989.
- Britton, G., Structure and nomenclature, in *Carotenoids in Photosynthesis*, Young, A. and Britton, G., Eds., Chapman & Hall, London, 1993, pp. 1–15.

- Britton, G., UV/visible spectroscopy, in *Carotenoids. Vol. 1B: Spectroscopy*, Britton, G., Liaaen-Jensen, S., and Pfander H., Eds., Birkhäuser Verlag, Basel, Switzerland, 1995a, chap. 2, pp. 13–62.
- Britton, G., Structure and properties of carotenoids in relation to function, *FASEB J.*, 9, 1551–1558, 1995b.
- Britton, G., Pigments in food: more than just colours, in *Pigments in Food Technology. Proceedings of the First International Congress in Food Technology*, Mínguez-Mosquera, M.I., Jarén-Galán, M., and Hornero-Méndez, D., Eds., PFT, Sevilla, Spain, 1999, pp. 3–9.
- Britton, G. and Hornero-Méndez, D., Carotenoids and colour in fruit and vegetables, in *Phytochemistry of Fruit and Vegetables*, Tomás-Barberán, F.A. and Robins, R.J., Eds., Clarendon Press, Oxford, 1997, pp. 11–27.
- Britton, G., Liaaen-Jensen, S., and Pfander, H., Carotenoids today and challenges for the future, in *Carotenoids. Vol. 1A: Isolation and Analysis*, Britton, G., Liaaen-Jensen, S., and Pfander H., Eds., Birkhäuser Verlag, Basel, Switzerland, 1995, chap. 2, pp. 13–26.
- Buchecker, R. and Noack, K., Circular dichroism, in *Carotenoids. Vol. 1B: Spectroscopy*, Britton, G., Liaaen-Jensen, S., and Pfander H., Eds., Birkhäuser Verlag, Basel, Switzerland, 1995, chap. 3, pp. 63–116.
- Bureau, J.L. and Bushway, R.J., HPLC determination of carotenoids in fruits and vegetables in the United-States, *J. Food Sci.*, 51, 128–130, 1986.
- Burton, G.W. and Ingold, K.U., Beta-carotene — an unusual type of lipid antioxidant, *Science*, 224, 569–573, 1984.
- Bushway, R.J., Separation of carotenoids in fruits and vegetables by high-performance liquid-chromatography, *J. Liq. Chromatogr.*, 8, 1527–1547, 1985.
- Bushway, R.J., Determination of α - and β -carotene in some raw fruits and vegetables by high-performance liquid chromatography, *J. Agric. Food Chem.*, 34, 409–412, 1986.
- Bushway, R.J. and Wilson, A.M., Determination of α - and β -carotene in fruits and vegetables by HPLC, *Can. Inst. Food Sci. Technol. J.*, 15, 165–169, 1982.
- Castenmiller, J.J.M. and West, C.E., Bioavailability and bioconversion of carotenoids, *Annu. Rev. Nutr.*, 18, 19–38, 1998.
- Chandler, A.L. and Schwartz, S.J., HPLC separation of *cis-trans* carotene isomers in fresh and processed fruits and vegetables, *J. Food Sci.*, 52, 669–672, 1987.
- Clevidence, B.A. and Bieri, J.G., Association of carotenoids with human plasma lipoproteins, *Methods Enzymol.*, 214, 33–46, 1992.
- Colditz, G.A., Branch, L.G., and Lipnick, R.J., Increased green and yellow vegetable intake and lowered cancer deaths in an elderly population, *Am. J. Clin. Nutr.*, 41, 32–36, 1985.
- Daood, H.G., Biacs, P.A., Hoshcke, A., Harkay-Vinkler, M., and Hajdu, F., Separation and identification of tomato fruit pigments by TLC and HPLC, *Acta Alimentaria*, 16, 339–350, 1987.
- Davies, B.H., Carotenoids, in *Chemistry and Biochemistry of Plant Pigments*, Vol. 2, Goodwin, T.W., Ed., Academic Press, New York, 1976, pp. 38–165.
- Davies, B.H. and Köst, H.P., Carotenoids, in *Handbook of Chromatography*, Vol.1, Köst, H.P., Ed., CRC Press, Boca Raton, FL, 1988.
- Davies, B.H., Matthews, S., and Kirk, J.T.O., The nature and biosynthesis of the carotenoids of different colour varieties of *Capsicum annuum*, *Phytochemistry*, 9, 797–805, 1970.
- Downham, A. and Collins, P., Colouring our foods in the last and next millennium, *Int. J. Food Sci. Technol.*, 35, 5–22, 2000.
- Englert, G., NMR spectroscopy, in *Carotenoids. Vol. 1B: Spectroscopy*, Britton, G., Liaaen-Jensen, S., and Pfander H., Eds., Birkhäuser Verlag, Basel, Switzerland, 1995, chap. 6, pp. 147–260.

- Enzell, C.R. and Back, S., Mass spectrometry, in *Carotenoids. Vol. 1B: Spectroscopy*, Britton, G., Liaaen-Jensen, S., and Pfander H., Eds., Birkhäuser Verlag, Basel, Switzerland, 1995, chap. 7, pp. 261–320.
- Eugster, C.H., History: 175 years of carotenoid chemistry, in *Carotenoids. Vol. 1A: Isolation and Analysis*, Britton, G., Liaaen-Jensen, S., and Pfander H., Eds., Birkhäuser Verlag, Basel, Switzerland, 1995, chap. 1, pp. 1–12.
- Everett, S.A., Dennis, F., Patel, K.B., Maddix, S., Kundu, S.C., and Willson, R.L., Scavenging of nitrogen dioxide, thiyl, and sulfonyl free radicals by the nutritional antioxidant β -carotene, *J. Biol. Chem.*, 271, 3988–3994, 1996.
- FAO/WHO Joint Expert Committee on Food Additives, *WHO Tech. Rep. Ser.*, 362, 1967.
- Farin, D., Ikan, R., and Gross, J., The carotenoid pigments in the juice and flavedo of mandarin hybrid (*Citrus reticulata*) cv michal during ripening, *Phytochemistry*, 22, 403–408, 1983.
- Ferruzzi, M.G., Sander, L.C., Rock, C.L., and Schwartz, S.J., Carotenoid determination in biological microsamples using liquid chromatography with a coulometric electrochemical array detector, *Anal. Biochem.*, 256, 74–81, 1998.
- Fisher, C. and Kocis, J.A., Separation of paprika pigments by HPLC, *J. Agric. Food Chem.*, 35, 55–57, 1987.
- Foppen, F.H., Tables for identification of carotenoid pigments, *Chromatogr. Rev.*, 14, 133–303, 1971.
- Frank, H.A. and Cogdell, R.J., The photochemistry and function of carotenoids in photosynthesis, in *Carotenoids in Photosynthesis*, Young, A. and Britton, G., Eds., Chapman & Hall, London, 1993, chap. 8, pp. 252–326.
- Gann, P.H., Ma, J., Giovannucci, E., Willett, W., Sacks, F.M., Hennekens, C.H., and Stampfer, M.J., Lower prostate cancer risk in men with elevated plasma lycopene levels: results of a prospective analysis, *Cancer Res.*, 59, 1225–1230, 1999.
- Gibson, G.R. and Fuller, R., The role of probiotics and prebiotics in the functional food concept, in *Functional Foods. The Consumer, the Products and the Evidence*, Sadler, M.J. and Saltmarsh, M., Eds., The Royal Society of Chemistry, Cambridge, 1998, pp. 3–4.
- Gibson, G.R. and Roberfroid, M.B., Dietary modulation of the human colonic microbiota — introducing the concept of prebiotics, *J. Nutr.*, 125, 1401–1412, 1995.
- Goodwin, T.W., Distribution of carotenoids, in *Chemistry and Biochemistry of Plant Pigments*, Vol. 1, Goodwin, T.W., Ed., Academic Press, New York, 1976, pp. 225–261.
- Goodwin, T.W., *The Biochemistry of Plant Pigments, Vol. 1*, Goodwin, T.W., Ed., Chapman & Hall, London, 1980.
- Goodwin, T.W. and Britton, G., Distribution and analysis of carotenoids, in *Plant Pigments*, Goodwin, T.W., Ed., Academic Press, London, 1988, pp. 61–132.
- Goodwin, T.W. and Goad, L.J., Carotenoids and triterpenoids, in *The Biochemistry of Fruits and Their Products*, Vol. 1, Hulme, A.C., Ed., Academic Press, New York, 1970, pp. 305–368.
- Gross, J., A rapid separation of citrus carotenoids by thin-layer chromatography, *Chromatographia*, 13, 572–573, 1980.
- Handelman, G.J., Carotenoids as scavengers of active oxygen species, in *Handbook of Antioxidants*, Cadenas, E. and Packer, L., Eds., Marcel Dekker, New York, 1996, chap. 10, pp. 259–313.
- Heinonen, M.I., Ollilainen, V., Linkola, E.K., Varo, P.T., and Koivistoinen, P.E., Carotenoid in Finnish foods: vegetables, fruits and berries, *J. Agric. Food Chem.*, 37, 655–659, 1989.
- Henry, B.S., Natural food colours, in *Natural Food Colorants*, Hendry, G.A.F. and Houghton, J.D., Eds., Blackie Academic and Professional, London, 1996, chap. 2, pp. 40–79.

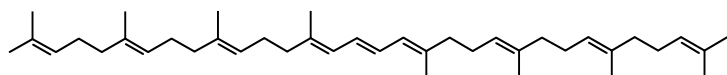
- Hornero-Méndez, D. and Mínguez-Mosquera, M.I., Xanthophyll esterification accompanying carotenoid overaccumulation in chromatoplast of *Capsicum annuum* ripening fruits is a constitutive process and useful for ripeness index, *J. Agric. Food Chem.*, 48, 1617–1622, 2000.
- Johjima, T. and Ogura, H., Analysis of tomato carotenoids by thin-layer chromatography and a cis-form γ -carotene newly identified in tangerine tomato, *J. Jpn. Soc. Hortic. Sci.*, 52, 202–209, 1983.
- Johnson, E.J., The role of lycopene in health and disease, *Nutr. Clin. Care*, 3, 35–43, 2000.
- Juhler, R.K. and Cox, R.P., High-performance liquid chromatographic determination of chloroplast pigments with optimized separation of lutein and zeaxanthin, *J. Chromatogr.*, 508, 232–235, 1990.
- Khachik, F., Beecher, G.R., Goli, M.B., and Lusby, W.R., Separation and quantification of carotenoids in foods, *Methods Enzymol.*, 213, 347–359, 1992a.
- Khachik, F., Beecher, G.R., Goli, M.B., Lusby, W.R., and Daitch, C.E., Separation and quantification of carotenoids in human plasma, *Methods Enzymol.*, 213, 205–219, 1992b.
- Khachik, F., Beecher, G.R., and Lusby, W.R., Separation, identification and quantification of the major carotenoids in extracts of apricots, peaches, cantaloupe and pink grapefruit by liquid chromatography, *J. Agric. Food Chem.*, 37, 1465–1473, 1989.
- Khachik, F., Beecher, G.R., and Whittaker, N.F., Separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography, *J. Agric. Food Chem.*, 334, 603–616, 1986.
- Koyama, Y., Resonance raman spectroscopy, in *Carotenoids. Vol. 1B: Spectroscopy*, Britton, G., Liaaen-Jensen, S., and Pfander H., Eds., Birkhäuser Verlag, Basel, Switzerland, 1995, chap. 5, pp. 135–146.
- Krinsky, N.I., Membrane antioxidants, *Ann. N.Y. Acad. Sci.*, 551, 17–33, 1988.
- Krinsky, N.I., Cornwell, D.G., and Oncley, J.L., The transport of vitamin A and carotenoids in human plasma, *Arch. Biochem. Biophys.*, 73, 233–246, 1958.
- Lee, C.Y., Changes in carotenoid content of carrots during growth and post-harvest storage, *Food Chem.*, 20, 285–293, 1986.
- Lee, C.Y., McCoon, P.E., and LeBowitz, J.M., Vitamin A value of sweet corn, *J. Agric. Food Chem.*, 29, 1294–1295, 1981.
- Lee, W.L., *Carotenoproteins in Animal Coloration*, Lee, W.L., Ed., Dowdens, Hutchinson & Ross, Stoudsburg, PA, 1977.
- Lesellier, E., Marty, C., Berset, C., and Tchaplal, A., Optimization of the isocratic non-aqueous reverse phase (NARP) HPLC separation of *trans/cis* α - and β -carotenes, *J. High Res. Chromatogr.*, 12, 447–454, 1989.
- Liebler, D.C., Antioxidant reactions of carotenoids, *Ann. N.Y. Acad. Sci.*, 669, 20–31, 1993.
- Mantoura, R.F.C. and Llewellyn, C.A., The rapid determination of algal chlorophyll and carotenoid pigments and their breakdown products in natural waters by reverse-phase high-performance liquid chromatography, *Anal. Chim. Acta*, 151, 297–314, 1983.
- Martin, H.D., Jäger, C., Ruck, C., Schmidt, M., Walsh, R., and Paust, J., Anti- and prooxidant properties of carotenoids, *J. Prakt. Chem.*, 341, 302–308, 1999.
- Mathews-Roth, M.M., Antitumor activity of beta-carotene, canthaxanthin and phytoene, *Oncology*, 39, 33–37, 1982.
- Mínguez-Mosquera, M.I. and Garrido-Fernández, J., Lipids removal during the extraction phase of chloroplast pigments in olive fruit (*Olea europaea*), *Grasas Aceites*, 36, 376–381, 1985.
- Mínguez-Mosquera, M.I., and Garrido-Fernández, J., Chlorophyll and carotenoid presence in olive fruit (*Olea europaea*), *J. Agric. Food Chem.*, 37, 1–7, 1989.

- Mínguez-Mosquera, M.I. and Hornero-Méndez, D., Separation and quantification of the carotenoid pigments in red peppers (*Capsicum annum*, L.), paprika and oleoresin by reversed-phase HPLC, *J. Agric. Food Chem.*, 41, 1616–1620, 1993.
- Mínguez-Mosquera, M.I., Garrido-Fernández, J., and Pereda-Marín, J., Paprika (*Capsicum annum*). Ratio between the red and yellow carotenoid pigments, *Grasas Aceites*, 35, 4–10, 1984.
- Mínguez-Mosquera, M.I., Jarén-Galán, M., Hornero-Méndez, D., Garrido-Fernández, J., Gallardo-Guerrero, M.L., and Gandul-Rojas, B., Decoloration of vegetable oils and oleoresins with recovery of unaltered pigments, *J. Am. Oil Chem. Soc.*, 68, 809–813, 1991a.
- Mínguez-Mosquera, M.I., Gandul-Rojas, B., Montañó-Asquerino, A., and Garrido-Fernández, J., Determination of chlorophylls and carotenoids by HPLC during olive lactic fermentation, *J. Chromatogr.*, 585, 259–266, 1991b.
- Moon, R.C., Comparative aspects of carotenoids and retinoids as chemopreventive agents for cancer, *J. Nutr.*, 119, 127–134, 1989.
- Olson, J.A., Vitamin A and carotenoids as antioxidants in a physiological context, *J. Nutr. Sci. Vitaminol.*, 39, S57–S65, 1993.
- O’Neil, C.A., Schwartz, S.J., and Catignani, G.L., Comparison of liquid chromatographic methods for determination of *cis-trans* isomers of β -carotene, *J. Assoc. Off. Anal. Chem.*, 74, 36–42, 1991.
- Oshima, S., Sakamoto, H., Ishiguro, Y., and Terao, J., Accumulation and clearance of cap-santhin in blood plasma after the ingestion of paprika juice in men, *J. Nutr.*, 127, 1475–1479, 1997.
- Philip, T. and Chen, T.S., Development of a method for the quantitative estimation of provitamin A carotenoids in some fruits, *J. Food Sci.*, 53, 1703–1706, 1988.
- Quackenbush, F.W., Reverse phase HPLC separation of *cis*-carotenoids and *trans*-carotenoids and its application to beta-carotenes in food materials, *J. Liq. Chromatograph.*, 10, 643–653, 1987.
- Quackenbush, F.W. and Smallidge, R.L., Nonaqueous reverse phase liquid-chromatographic system for separation and quantitation of provitamins-A, *J. Assoc. Off. Anal. Chem.*, 69, 767–772, 1986.
- Rodríguez-Amaya, D.B., Critical review of provitamin A determination in plant foods, *J. Micronutr. Anal.*, 5, 191–225, 1989.
- Saleh, M.H. and Tan, B., Separation and identification of *cis,trans* carotenoids isomers, *J. Agric. Food Chem.*, 39, 1438–1443, 1991.
- Sander, L.C., Sharpless, K.E., and Pursch, M., C_{30} stationary phases for the analysis of food by liquid chromatography, *J. Chromatogr.*, A 880, 189–202, 2000.
- Sandmann, G., Albrecht, M., Schnurr, G., Knörzer, O., and Böger, P., The biotechnological potential and design of novel carotenoids by gene combination in *Escherichia coli*., *Trends Biotechnol.*, 17, 233–237, 1999.
- Schiedt, K., Isolation and analysis, in *Carotenoids. Vol. 3: Biosynthesis*, Britton, G., Liaaen-Jensen, S., and Pfander, H., Eds., Birkhäuser Verlag, Basel, Switzerland, 1998, chap. 7, pp. 285–355.
- Schiedt, K. and Liaaen-Jensen, S., Isolation and analysis, in *Carotenoids. Vol. 1A: Isolation and Analysis*, Britton, G., Liaaen-Jensen, S., and Pfander, H., Eds., Birkhäuser Verlag, Basel, Switzerland, 1995, chap. 5, pp. 81–108.
- Scolnik, P.A. and Bartley, G.E., A table of some cloned plant genes involved in isoprenoid biosynthesis, *Plant Mol. Biol. Rep.*, 14, 305–319, 1996.
- Sherma, J., Thin-layer chromatography in food and agricultural analysis, *J. Chromatogr.*, A, 880, 129–147, 2000.

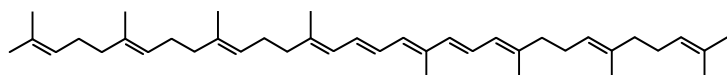
- Sitte, P., Falk, H. and Liedvogel, B., Chromoplasts, in *Pigments in Plants*, Czygan, F.-Ch., Ed., Gustav Fischer, Stuttgart, 1980, pp. 117–148.
- Speek, A.J., Temalilwa, C.R., and Schrijver, J., Determination of beta-carotene content and vitamin-a activity of vegetables by high-performance liquid-chromatography and spectrophotometry, *Food Chem.*, 19, 65–74, 1986.
- Stahl, E., *Thin Layer Chromatography. A Laboratory Handbook*, Springer-Verlag, Berlin, 1967.
- Stahl, W. and Sies, H., Perspectives in biochemistry and biophysics. Lycopene: a biological important carotenoid for humans? *Arch. Biochem. Biophys.*, 336, 1–9, 1996.
- Stewart, I. and Wheaton, T.A., Continuous separation of carotenoids by liquid chromatography, *J. Chromatogr.*, 55, 325–336, 1971.
- Strain, J.J. and Benzie, I.F.F., Antioxidant nutrients, in *Functional Foods. The Consumer, the Products and the Evidence*, Sadler, M.J. and Saltmarsh, M., Eds., The Royal Society of Chemistry, Cambridge, 1998, pp. 74–79.
- Swanson, J.E. and Parker, R.S., Biological effects of carotenoids in humans, in *Handbook of Antioxidants*, Cadenas, E. and Packer, L., Eds., Marcel Dekker, New York, 1996, chap. 12, pp. 337–367.
- Terao, J., Antioxidant activity of β -carotene-related carotenoids in solution, *Lipids*, 24, 659–661, 1989.
- Thompson, J.N., Hatina, G., and Maxwell, W.B., High performance liquid chromatographic determination of vitamin A in margarine, milk, partially skimmed milk and skimmed milk, *J. Assoc. Offic. Anal. Chem.*, 63, 894–898, 1980.
- Tsukida, K., Saiki, K., Takii, T., and Koyama, Y., Separation and determination of *cis,trans* β -carotenes by HPLC, *J. Chromatogr.*, 245, 359–364, 1982.
- Tswett, M., Adsorptionsanalyse und chromatographische method: Anwendung auf die Chemie des Chlorophylls, *Berl. Dtsch. Bot. Ges.*, 24, 384–393, 1906.
- Van Breemen, R.B., Innovations in carotenoid analysis using LC/MS, *Anal. Chem.*, 68, 299A–304A, 1996.
- Van het Hof, K.H., West, C.E., Weststrate, J.A., and Hautvast, J.G., Dietary factors that affect the bioavailability of carotenoids, *J. Nutr.*, 130, 503–506, 2000.
- Vetter, W., Englert, G., Rigassi, N., and Schwieter, U., Spectroscopic methods, in *Carotenoids*, Isler, O., Ed., Birkhäuser Verlag, Basel, Switzerland, 1971, chap. 4, pp. 189–266.
- Weedon, B.C.L., Occurrence, in *Carotenoids*, Isler, O., Ed., Birkhäuser Verlag, Basel, Switzerland, 1971, chap. 2, pp. 29–59.
- Weedon, B.C.L. and Moss, G.P., Structure and nomenclature, in *Carotenoids. Vol. 1A: Isolation and Analysis*, Britton, G., Liaaen-Jensen, S., and Pfander H., Eds., Birkhäuser Verlag, Basel, Switzerland, 1995, chap. 3, pp. 27–70.
- Zagalsky, P.F., Invertebrate carotenoproteins, *Methods Enzymol.*, 111, 216–247, 1985.
- Zechmeister, L., Stereoisomeric provitamins A., *Vitam. Horm.*, 7, 57–81, 1949.
- Zhang, L.X., Conney, R.V., and Bertram, J.S., Carotenoids enhance gap junctional communication and inhibit lipid peroxidation in C3H/10T1/2 cells: relationship to their cancer chemopreventive action, *Carcinogenesis*, 12, 2109–2114, 1991.
- Ziegler, R.G., A review of epidemiologic evidence that carotenoids reduce the risk of cancer, *J. Nutr.*, 119, 116–122, 1989.

APPENDIX 3.I

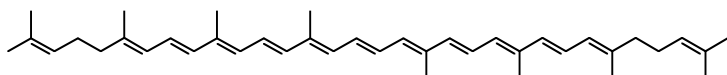
CHEMICAL STRUCTURES FOR CAROTENES AND XANTHOPHYLLS COMMONLY FOUND IN FOODS



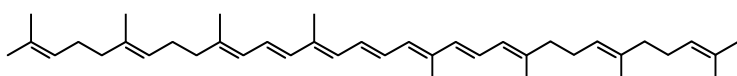
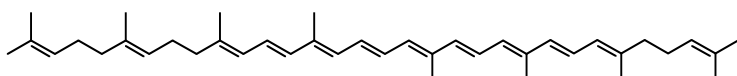
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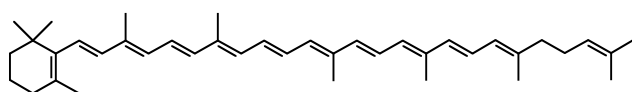
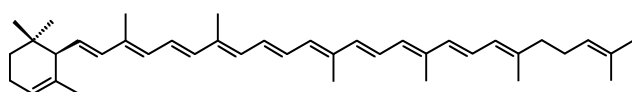
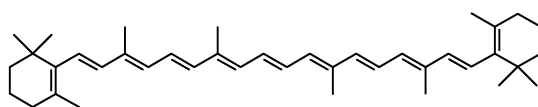
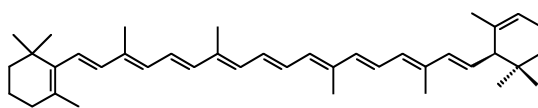
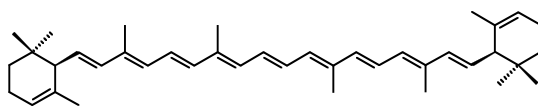
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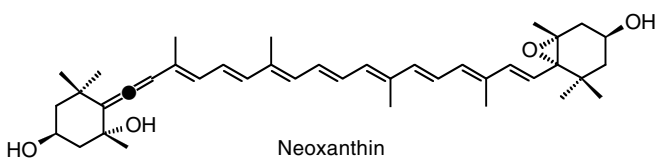
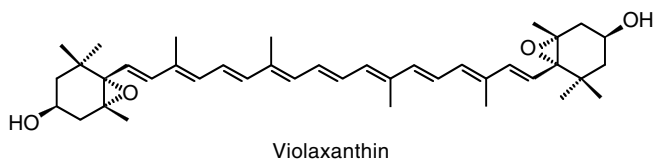
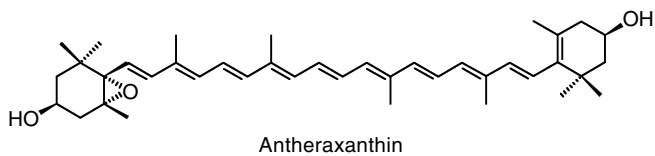
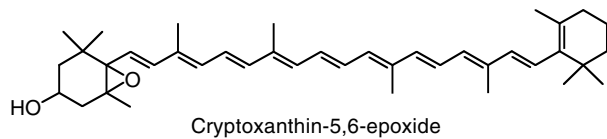
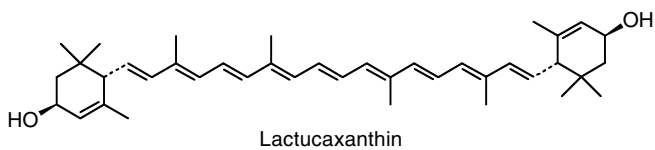
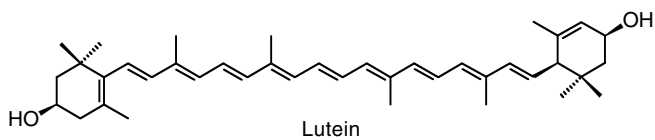
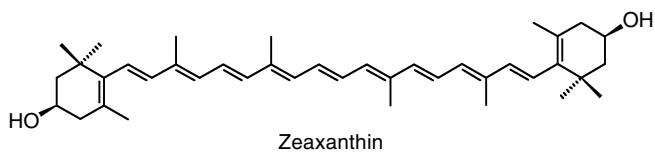
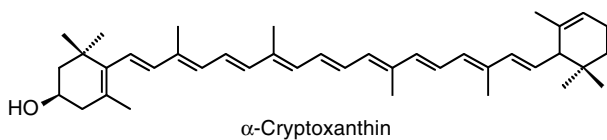
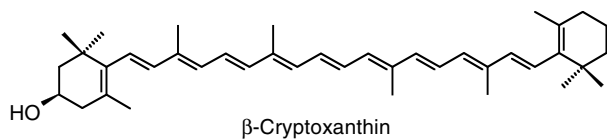


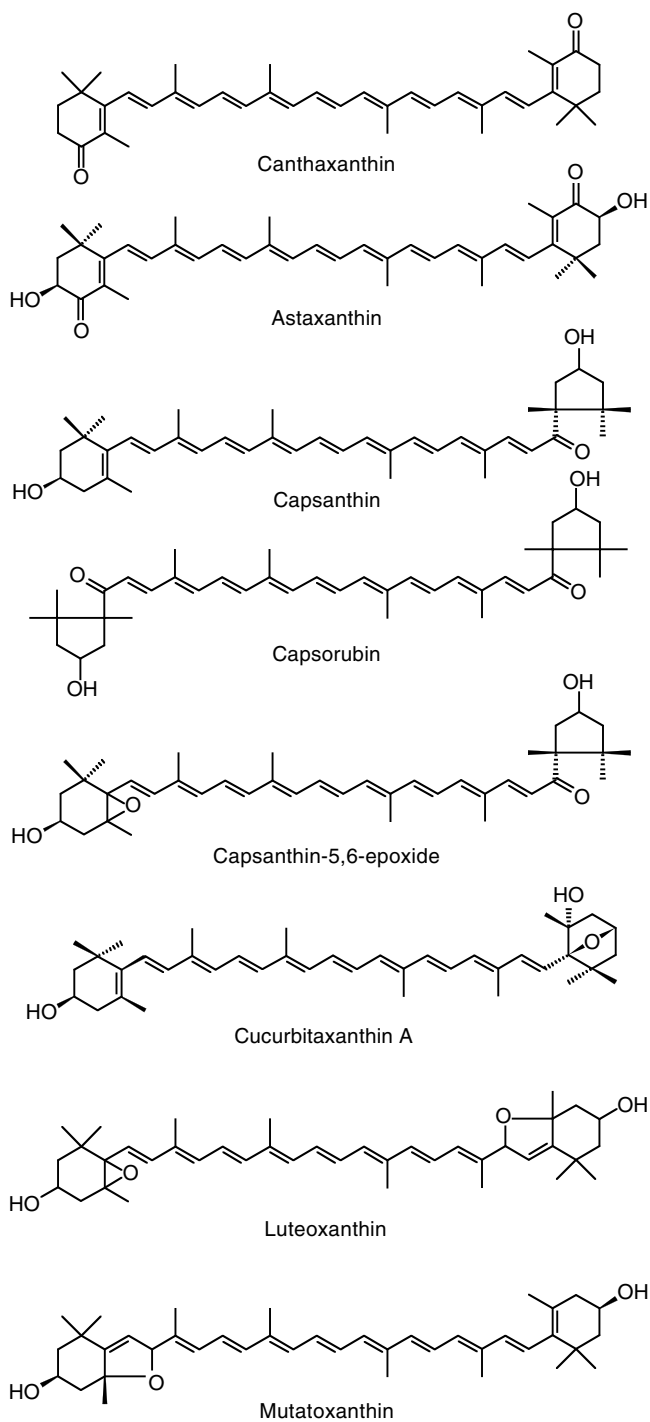
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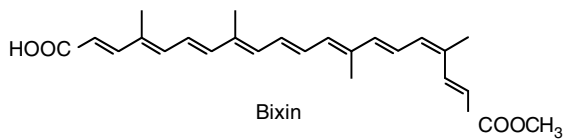
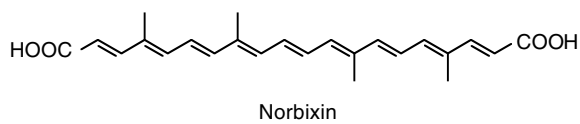
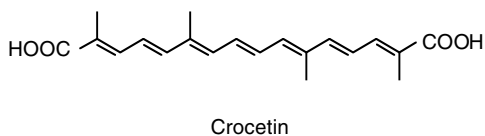
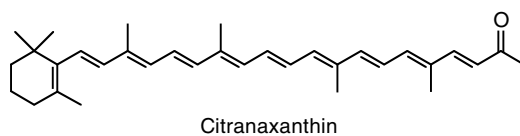
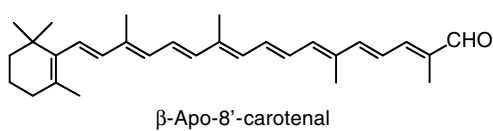
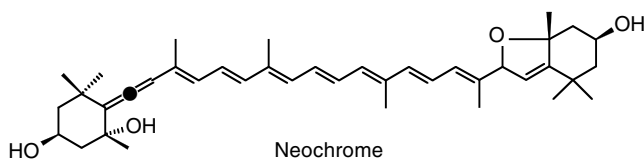
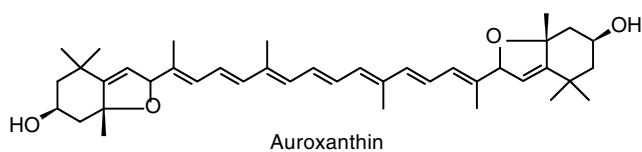
 ζ -Carotene

Neurosporene

 γ -Carotene δ -Carotene β -Carotene α -Carotene ϵ -Carotene







4 Chlorophylls

*M. Isabel Mínguez-Mosquera,
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and Manuel Jarén-Galán*

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4.1 INTRODUCTION

Numerous components present in our normal diet cannot be considered nutrients or drugs, but they are on the dividing line between the two. Their absence from the diet does not cause a nutritional deficit, but their presence, above all those of plant origin, helps to prevent certain diseases in the long term.

Components of our diet are normally grouped in five great families: carbohydrates, lipids, proteins, oligoelements, and vitamins, with each having a particular biological function in nutrition. Besides these basic nutrients, there are many other minor constituents that are not members of these great families, such as chlorophylls, flavonoids, coumarins, etc. They are not essential for correct nutrition and are therefore not nutrients, and they are not considered substances having direct therapeutic activity, although some do at concentrations much higher than those found in our normal diet. However, when foods containing these compounds are a regular part of the diet, the incidence and prevalence of certain diseases are changed.

The popularization of advances in knowledge about the properties of dietary components has given rise to a more rational consumer culture. Technology guarantees that any consumer product meets hygiene-health standards in nutritional function, and consumers are now more interested in quality and the preservation of the whole potential of the foodstuff, conceiving it as rather more than a simple source of proteins or carbohydrates. From the technological and consumer viewpoint, this is a culture in which food serves not only for replacement and to repair wear and tear of the organism, but can also help to prevent future disorders. It is the beginning of consumption based on the interrelationship between product quality and food quality.

Knowing the functional activity of certain compounds means that their presence in foods is valued positively and gives the consumer an additional parameter for product choice. Compounds whose presence is appreciated possibly for their esthetic function or as indicators of the state of ripeness and technological treatment of the foodstuff, as is the specific case of the chlorophylls, are given new scope.

We use our visual sense unconsciously to judge the quality of a product. We expect a fresh vegetable to be bright green, the color of chlorophylls, while one subjected to fermentation will be olive green, typical of pheophytins and pheophorbides. Colors other than those expected make us suspect the quality of the product, and analysis will corroborate the presence of chlorophyll transformation compounds

improper of such product. The initial judgment of product quality, perhaps without our knowing it, depends on the pigments responsible for the color. Chlorophyll pigments have a double role in alimentation: esthetic and technological. Being pigments, and possessing chromatic properties, they make a product attractive and appetizing and give important visual and analytical information about the history of the product. However, chlorophyll compounds are not inert components in our diet; they fulfill certain biological functions that are effective as long as the chlorophyll derivative conserves the basic porphyrin ring structure. Thus, the presence of chlorophylls and their transformation products contribute added value to the plant product.

Although there has been no deep research into the role of chlorophyll compounds in the diet, there are numerous epidemiological studies on the incidence of certain types of cancer between populations with similar dietary habits, but they differ essentially in the ingestion of chlorophyll-rich plant food. Statistically, there seems to be a relationship between high intake of chlorophyll in the diet and a decreased risk of certain types of cancer. However, the possibility cannot be excluded that the factor responsible for the anti-cancer activity is some other compound not yet detected.

Although the epidemiological results indicate a relationship between the ingestion of chlorophyll-rich plants and a decreased risk of certain types of cancer, error in assuming cause-effect cannot be ruled out. In experiments *in vitro*, the relationship is clear and overwhelming. The chlorophyll compounds are able to inhibit the genotoxic action of certain substances, particularly those with an aromatic structure, and act as protectors against ionizing radiation, due to a high affinity in the link between DNA and the porphyrin ring of the chlorophyll molecule.

This seems to indicate that, in fact, the role of dietary chlorophyll compounds is perhaps not as nutrients in the strict sense, since they are not necessary for our proper physiological functioning, but to prevent disorders and as protectors against aggression. Currently, the study of these compounds in our diet is moving away from purely technological aspects, including chromatic properties and use as quality indicators, toward an area midway between nutrition and pharmacology. From the alimentary point of view, the presence of chlorophyll compounds in green plants is important in every possible sense. We require that the food be visually attractive and its color appropriate for the treatment received and that we know beforehand its activity in our organism, so that we can make the best judgment of the product.

Probably without our being conscious of the fact, the chlorophyll compounds that are a regular and widespread part of the human diet have an important protective role. A much deeper study is required of these compounds, which are not essential in our nutrition and whose lack does not mean alterations in the physiological functions, but which do contribute to protection against toxic compounds.

4.2 GENERAL ASPECTS

4.2.1 STRUCTURE, LOCATION, FUNCTION, AND DISTRIBUTION

Numerous works carried out in the last 100 years have elucidated the chlorophyll structure. Willstatter, in 1906, was the first to achieve separation of chlorophyll *a* in

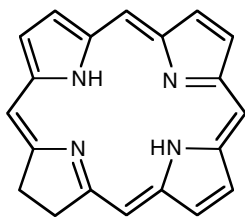


FIGURE 4.1 Structure of porphyrin.

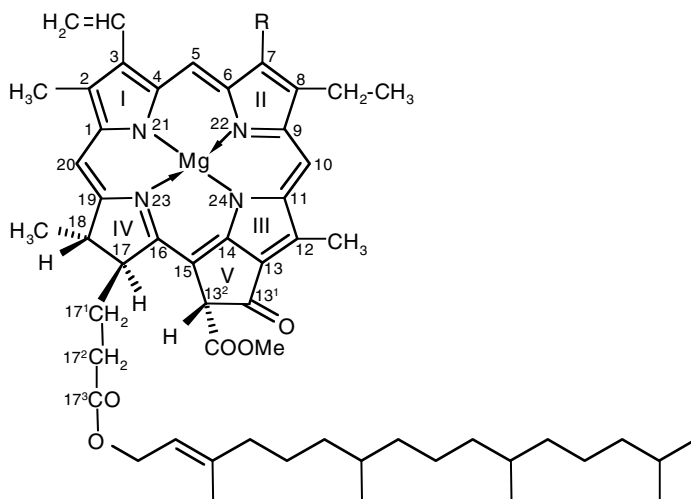


FIGURE 4.2 Structures of chlorophyll *a* ($R = \text{CH}_3$) and chlorophyll *b* ($R = \text{CHO}$).

pure state (Vernon and Seely, 1966). Later, its structure was established by degradation studies and proved by total synthesis.

Chemically, chlorophyll is classified within the porphyrin group. The common structure of this series of compounds is porphyrin, comprising four units of pyrrole, whose α -positions are linked by methine bridges, forming a new planar aromatic system that is very stable (Figure 4.1). The most noteworthy characteristic of the porphyrins is their readiness to form chelates (intramolecular bonds) with metal ions, leaving the metal firmly bonded in the space bounded by the four nitrogen atoms in the planar system. This basic structure comprises 11 conjugated double bonds making up the chromophore group, which is able to absorb radiation in the visible spectrum. The porphyrins are generally brightly colored; the basic porphyrin system is orangey, with the color of the porphyrin compound depending on the nature of both the ring substituents and the central atom.

In the case of chlorophyll, the metal ion forming the complex is magnesium (Mg) (Figure 4.2). Chlorophyll also contains a modified propionic acid chain in the form of cyclic β -ketoester (isocyclic ring) and, on C-17, a chain of propionic acid esterified with a diterpene alcohol of C_{20} structure, phytol, making the molecule

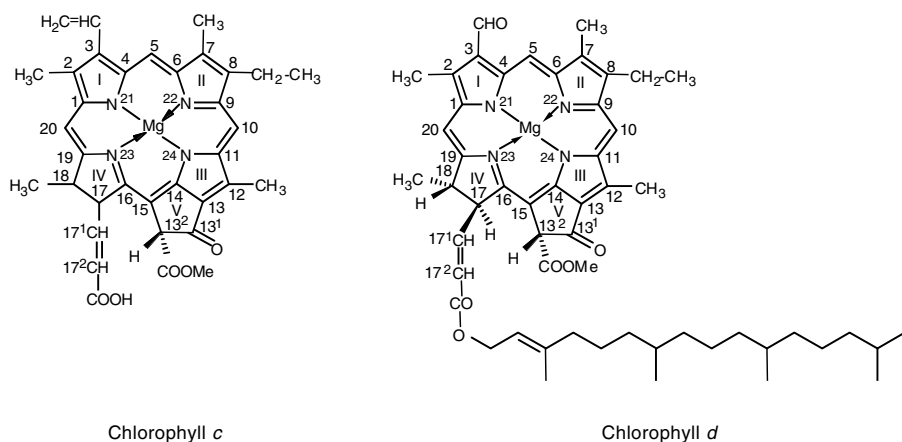


FIGURE 4.3 Structures of chlorophyll *c* and chlorophyll *d*.

liposoluble. The difference between chlorophylls *a* and *b* lies in that chlorophyll *a* has a methyl group on C-7, whereas chlorophyll *b* has a formyl group. Chlorophyll *c* has a residue of propenoic acid on C-17, and chlorophyll *d* lacks the unsaturation between carbons 17 and 18 of ring IV (Figure 4.3). In the bacteriochlorophylls, the alcohols that esterify on C-17³ are, apart from phytol, farnesil (C₁₂) and geranyl geraniol (C₁₆).

The chlorophyll pigments of higher plants are located, together with a series of carotenoids, in the plastids, which are differentiations of the endoplasmic reticulum, isolated from the rest of the cytoplasm by their own membranes and with their own genetic information. Plastids that have chlorophyll content, and thus photosynthetic capacity, are denominated chloroplasts. This organelle has a system of double membranes in the form of sacs — the thylakoids — which contain all the chlorophyll. Some are large and isolated (stroma thylakoids), and others are smaller and piled (grana thylakoids). Toward the interior of the chloroplast is a gelatinous and highly viscous phase, the stroma, which contains a group of enzymes, occasionally large granules of assimilation starch and small dark bodies, denominated plastoglobules, considered reservoirs of lipids (Strasburger, 1974).

The thylakoid membrane is a lipid bilayer composed of galactolipids and small amounts of phospholipids. The amphoteric nature of the chlorophylls allows the phytol chain to be embedded in the membrane, with the porphyrin ring linked by noncovalent bonds to proteins (Heaton and Marangoni, 1996; Salisbury and Ross 1985), constituting supermolecular structures known as *photosystems*. In higher plants, there are always two photosystems: PSI and PSII. PSII is responsible for photolysis of water, and PSI is responsible for the reduction of NADP⁺. It is noteworthy that chlorophyll *b* is found mainly in PSII, exclusive to organisms with oxygenic photosynthesis, and is evolutionarily more developed (Gross, 1987).

Chlorophyll–protein interaction enables the capture of radiation energy within the whole visible range, because the protein bonding modifies the excitation energy. Consequently, up to six types of chlorophyll *a* protein grouping have been found *in*

vivo, with characteristic electron absorption maxima (Cla661, Cla669, Cla677, Cla684, Cla691 and Cla700 to Cla720). In the case of chlorophyll *b*, only two groupings have been found (Clb640 and Clb650) (Gross, 1987).

It is difficult to conceive the origin or existence of life without the presence of photosynthetic pigments able to absorb radiation energy and transform it into chemical energy. The whole process is based on the existence of photoreceptive molecules, the chlorophylls, which translate light energy into chemical energy. The set of reactions occurring from the photoexcitation of the chlorophylls until the storage of the light energy in the form of sugars is denominated "photosynthesis" (Devlin, 1975).

All photosynthetic cells contain one or more types of pigment, but not all are green. The photosynthetic algaes and the bacteriae can be brown, red, or purple. This variety of colors is because, besides chlorophyll, many photosynthetic cells contain other pigments, called accessories, that act as supplementary receptors of light for portions of the visible spectrum that are not covered completely by chlorophyll, thus cooperating in the transfer of light energy. These are the yellow carotenoids, the blue phycocyanins, and the red phycoerythrins. The carotenoids also protect chlorophyll from the degradative attack of molecular oxygen produced during the photosynthetic process.

As chlorophyll *a* is common to all photosynthetic organisms, it has been postulated that it is the only pigment supplying energy directly to the photosynthetic reaction and that all the others transfer the absorbed energy to chlorophyll *a* (Jackson, 1976; Stainer et al., 1965).

It is not easy to define how plant aging affects the pigments. Certainly, there is a sequence of transformations during fruit ripening, accompanied by changes in flavor, texture, color, etc. However, many fruits are chlorophyll free before ripeness, with the chloroplasts replaced by chromoplasts. The degradation of chlorophylls during the biosynthesis of carotenoids and/or anthocyanins and betalains is complex (Simpson et al., 1976; Strasburger, 1974). As the fruits approach ripeness, there is a stage in which biochemical changes are initiated by the autocatalytic production of ethylene. This increase in respiration marks the point between development and ripening, with a variation in fruit skin coloration (Bauernfeind et al., 1971). The yellow of autumn leaves in many plants is the result of the elimination (by the conductor routes) of chlorophylls that have decomposed, while the carotenoids remain. The same does not happen with the red coloration of autumn leaves. In this case, the cell fluid is tinted by anthocyanins. In general, the yellow or orangey color of most ripe fruits, such as lemons and oranges (genus *Citrus*), is due to an enrichment in carotenoid pigments accompanied by the disappearance of chlorophyll.

Chlorophylls *a* and *b* are widely distributed in nature and are the best-known types. Chlorophyll *a* is found in all photosynthetic organisms except some groups of bacteriae. Chlorophyll *b* is present in all higher plants and in algaes of the divisions Chlorophyta and Euglenophyta, always accompanying chlorophyll *a* as an accessory pigment in the photosynthetic process. Other chlorophyll types (*c*, *d*, and *e*) are found only in algaes, always in combination with chlorophyll *a*. The bacteriochlorophylls are the chlorophylls of photosynthesizing bacteriae and structurally are slightly different to those typical of higher plants.

In higher plants, only chlorophylls *a* and *b* are present. Their ratio normally varies between 3 and 1, depending on a multitude of factors — both genetic (species, variety, etc.) and environmental (luminosity, water stress, mineral nutrition, etc.) (Lichtenthaler, 1968). Thus, for instance, plants exposed to the sun tend to have higher *a* to *b* ratio than plants in the shade. The chlorophylls generally contribute 0.6 to 1.2% of dry weight (Willstatter and Stoll, 1928).

4.2.2 CHEMICAL PROPERTIES

4.2.2.1 Structural Modification of Chlorophyll

In their natural location within the chloroplast, the chlorophylls are quite stable compounds, but when this organelle is damaged and loses its physiological elements, they become extremely labile, susceptible to a wide range of structural modifications by factors such as temperature, pH, enzyme action, molecular oxygen, and light.

There are four points at which the main structural modifications of the chlorophyll molecule start: (1) the chelate, (2) the ester bond of the phytol alcohol (C-17³), (3) the isocyclic ring (C-13²), and (4) the basic porphyrin structure. Figure 4.4 shows the possible transformations of the chlorophyll molecule.

4.2.2.1.1 Chelate Modification

The reaction substituting the coordination ion, Mg, by two hydrogen (H) atoms is known as pheophytinization. It modifies the properties of the chromophore, and the resulting compound has a different color. In the case of chlorophyll *a*, which is bluish-green, grey pheophytin *a* is formed, and in that of chlorophyll *b*, the yellow-green color turns to the brown of pheophytin *b*. There is evidence that *in vivo* the enzyme Mg dechelataase catalyzes this reaction, playing an important role in chlorophyll catabolism (Heaton and Marangoni, 1996; Langmeiner et al., 1993), although its existence has yet to be confirmed. Chemically, this reaction takes place under acid conditions. Thus, there will be pheophytins whenever there is loss of cell integrity and acids are liberated. All the kinetic studies of the reaction agree that the rate of pheophytinization of chlorophyll *a* is always higher than that of chlorophyll *b*: between 2.5- and 10-fold (Mínguez-Mosquera et al., 1994a; Schwartz and Lorenzo, 1990; Weemaes et al., 1999).

The substitution of H by another divalent metal originates a new coordination complex, which may be even more stable than the original Mg chelate, modifying the characteristics of the chromophore. If the substitution is by copper (Cu) or zinc (Zn), the complex recovers the greenish color and is more stable (Inoue et al., 1988, 1993; Schaber et al., 1984; Schwartz, 1984). As with the former reaction, kinetic studies show a higher rate of formation of the metal complex for pheophytin *a* than for pheophytin *b*. The formyl group present on C-7 of the *b*-series derivatives supplies a salient electron to the substituent, resulting in a decrease in electron density on the pyrrole nitrogens and thus a lower reactivity with the metal cations. At the same time, it seems that the phytol chain interferes sterically in the complexation reaction, with the highest rate of insertion of the metal ion in chlorophyll derivatives de-esterified at C-17³ (Tonnuci and von Elbe, 1992).

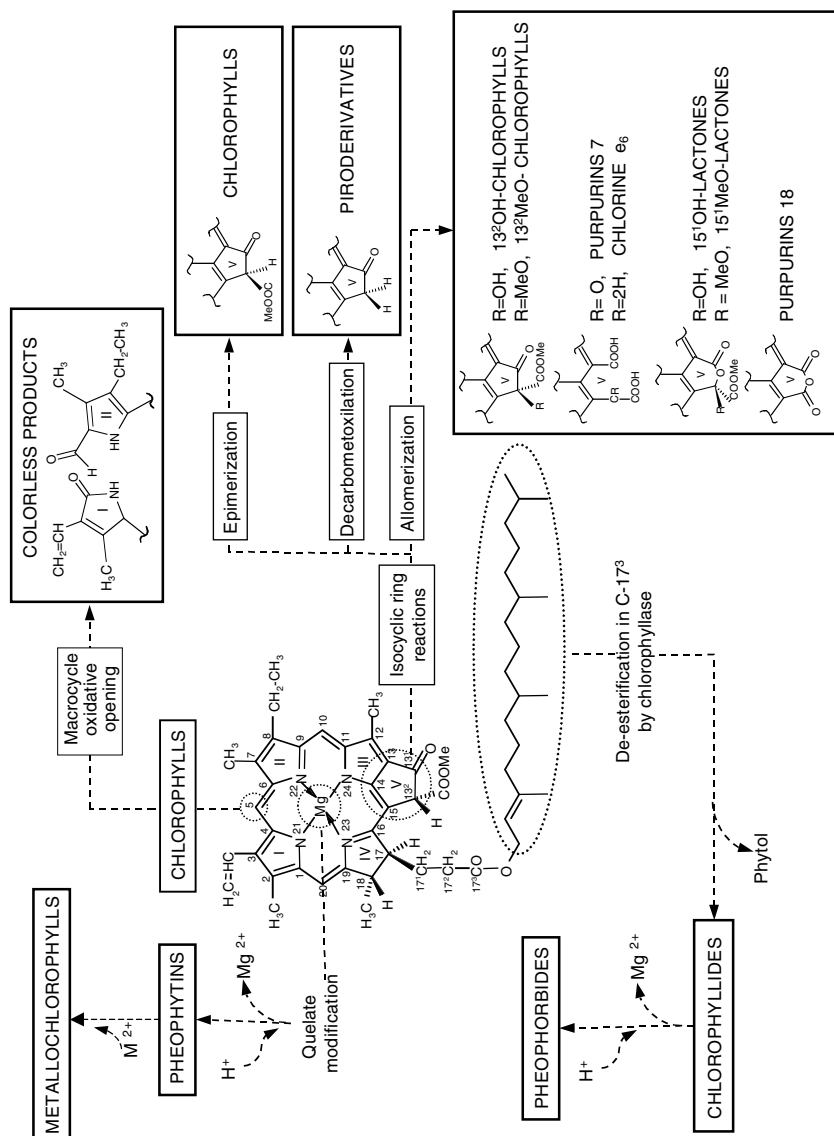


FIGURE 4.4 Transformations of the chlorophyll molecule.

The formation has also been described of other metallochlorophylls — Ni(II), Co(II), Fe(II) and Fe(III), Mn(II) and Mn(III), La(II) — which are of use in the pharmaceutical industry and in the chemotherapy of cancer (Furuya et al., 1987, 1988; Li and Inoue, 1992; Li et al., 1997; Nonomura et al., 1994). Resiting the Mg atom in the porphyrin ring is difficult, except with the use of the Grignard reagent.

4.2.2.1.2 *De-esterification at C-17³*

This is a specific reaction catalyzed by the endogenous enzyme chlorophyllase (chlorophyll-chlorophyllide-hydrolase) (EC 3.1.1.14), giving rise to chlorophyllides. The basic macrocyclic structure is not modified, so the chromophore properties are maintained and the color is unaltered. However, phytol (which makes the chlorophyll molecule liposoluble) is lost, resulting in a considerably increased polarity of the product. Pheophytin can also be the substrate of the enzyme chlorophyllase, with the reaction product of de-esterification being pheophorbide. Chemical de-esterification of phytol under acid or alkaline conditions is not a specific reaction and is usually accompanied by oxidative side-reactions (Mínguez-Mosquera and Gandul-Rojas, 1995)

4.2.2.1.3 *Reactions of the Isocyclic Ring*

Epimerization on C-13² — This is the mildest alteration possible. It is simply a spatial isomerization, and does not involve changes in the color or the electron absorption spectrum, only a slight increase in polarity of the molecule. These compounds are not found naturally in fresh plant tissue, although they are often detected as a result of solvent extraction processes. Polar solvents containing exchangeable hydrogens assist the formation of such derivatives, as do Lewis bases, which act as receptors of hydrogen ions from the porphyrin molecule. In general, and as in all reactions, this increases with temperature.

Decarbomethoxylation on C-13² — This is an oxidative reaction in which the carbomethoxy group of C-13² (COOCH₃) is substituted by H with no effect on the basic porphyrin structure. The compounds formed are denominated pyroderivatives and have the same coloration and spectroscopic properties as their precursors (chlorophylls, pheophytins, chlorophyllides, and pheophorbides), being differentiated only in a slight decrease in polarity. The progress of this reaction is governed by a combination of the factors time and temperature.

Allomerization — These are the reactions in which the isocyclic ring is oxidized by triplet molecular oxygen (³O₂). They include a complex series of oxidation on C-13², in which the H is substituted by an oxygen or oxygenated chemical species. If the oxidation takes place in aqueous medium, the substituent is an OH group, and 13²OH-chlorophyll is formed. If the reaction takes place in alcoholic solution, e.g., methanol, the substituent is a methoxyl group (MeO-) and originates 13²-MeO-chlorophyll. Different reaction mechanisms have been proposed; the most widely accepted is via free radicals, with the enolate anion as intermediary, although details of the reaction remain unknown. These reactions can also be catalyzed by oxidative enzymes such as chlorophyll-oxidase or peroxidases. No changes are shown in color or electron absorption spectrum, since none of them modifies the porphyrin ring, but there is a slight increase in polarity.

The opening of the isocyclic ring and the oxidation of C-13² originate the purpurins 7, which, according to their structure, are also denominated as 15¹-glyoxylic acid chlorophyll *a*. Although this reaction does not affect the basic macrocyclic structure, the cleavage of the isocyclic ring considerably modifies the electron absorption spectrum and the adsorption properties, as the compounds are much more polar.

In these oxidation reactions, the formation of a cyclic ester originates a lactone ring. Under mild oxidation conditions, OH-lactones of chlorophylls are formed, and under more severe conditions, the purpurins 18 originate. Although the basic chromophore structure is conserved, there are changes in the electron absorption spectrum and the polarity.

If the allomerization reaction takes place in a slightly alkaline medium and under inert atmosphere, there is only solvolysis of the isocyclic ring, and the resulting components are denominated esterified chlorin *e*₆ and rhodin *g*₇, depending on whether they come from the *a*- or *b*-series, respectively. Chlorophyll *a* yields Mg-phytyl-chlorin *e*₆, while pheophytin *a* or pheophorbide *a* yields obtained phytol-chlorin *e*₆ or free chlorin *e*₆, respectively. The same series of transformations can be described for chlorophyll *b*. The structural difference between these compounds and the purpurins 7 is solely in the substituent of C-15¹, which, in place of an O, is an H, so that their coloration, polarity, and electron absorption properties are very similar. Other techniques, such as mass spectrometry (MS), must be used for their differentiation (Mínguez-Mosquera et al., 1996).

4.2.2.1.4 Cleavage of the Macrocycle

This takes place in a series of oxidative reactions beginning with cleavage of the porphyrin ring, which loses its original chromophore properties, giving rise to colorless products. Cleavage begins at C-5, and the first derivatives maintain chromophore groups that emit fluorescence but do not absorb light in the visible spectrum (FC). There is some evidence that a dioxygenase is the enzyme responsible for this reaction (Ginsburg et al., 1994; Hörtensteiner et al., 1995; Schellenberg et al., 1993). Finally, these derivatives are converted into nonfluorescent products (RF) on losing the delocalized double bond system in the pyrrole rings (Ginsburg and Matile, 1993).

4.2.2.2 Functional Properties

Chlorophylls and their derivatives are a group of compounds with recognized biological activity that can take place at the levels of concentration normally found in the diet. Studies until now have focused essentially on the antimutagenic and antigenotoxic activity exhibited by all chlorophyll derivatives, including water-soluble salts (Lin, 1994).

The first studies relating chlorophylls with cancerous processes date from the 1930s to 1940s, when accumulations of porphyrin derivatives were detected in sarcomas and carcinomas of the breast. Current lines of research focus on the preventive activity exhibited by chlorophyll derivatives against tumor development.

An exhaustive review of the anticarcinogenic activity of chlorophyll compounds was published in 1997 by Dashwood. It seems clear, from the studies carried out,

that all chlorophyll derivatives have a chemoprotective activity against aromatic mutagenic agents, forming molecular complexes by ring overlapping. This possible mechanism of action would explain why all chlorophyll transformation derivatives, and even the synthetic water-soluble compounds obtained from the chlorophyll molecule, show chemoprotective activity.

The equilibrium constants that have been obtained for the reaction of formation of molecular complexes between different chlorophyll derivatives and mutagenic agents with aromatic heterocyclic structure suggest that the chlorophyll concentration levels necessary for effective protection against these agents are within the range usually reached in a normal and balanced diet.

In vivo studies using different administration routes for both carcinogen and protective chlorophyll show a marked disparity in protective activity of the chlorophyll compounds depending on the administration route. The highest effectiveness is achieved when the two substances are ingested together. A recent work carried out in *Drosophila* demonstrated that oral, joint administration of a mutagenic agent and a chlorophyll concentrate obtained from spinach and algaes suppressed the genotoxic activity of the mutagen (Negishi et al., 1997). Topical treatments also gave high protective activity. Tests *in vivo* and *in vitro* have demonstrated that the use of pheophytins *a* and *b* obtained from the nonphenolic fraction of green tea markedly suppressed the development of skin tumors in mice (Higashi-Okai et al., 1998). With oral or topical administration, the time of contact between the mutagen and the chlorophyll compound before they are absorbed is high, enabling the two to interact chemically, inactivating the carcinogenic capacity and consequently reducing the rate of tumor appearance. These results directly support the hypothesis of molecular complexation between the two species.

When the carcinogen is injected and the chlorophyll compounds are administered orally, the chemoprotective activity is low, probably because the carcinogen reaches its target cell before interacting with the chlorophyll compounds. In this case, there are also differences in the protective activity of the different chlorophyll compounds, possibly due to the different rate of intestinal absorption of each specific chlorophyll compound, depending on its structure and polarity. The results obtained are evidently insufficient, and in some cases contradictory, because during the passage of the chlorophyll compounds through the intestine, numerous coexisting transformation products are originated, making it difficult to assign a specific activity to a particular compound.

The chemoprotective activity against carcinogens has been used as a preventative in chemotherapy treatments of tumors. Most drugs used in tumor treatment can have side effects and induce genotoxic effects in healthy cells. Tests both *in vitro* (Gentile et al., 1998) and *in vivo* with mice (Te et al., 1997) have demonstrated the efficacy of chlorophyll derivatives in decreasing the collateral mutagenic activity of the drug in all cases without modifying the antitumoral effect.

When using drugs with collateral mutagenic capacity to treat cancer in human patients, food supplements are commonly administered to diminish the side effects of the therapy. Such foods are often rich in antimutagenic compounds, and for many years, chlorophylls, chlorins, and other porphyrins have been used in clinical treatments (Kumar et al., 1999).

4.2.3 SPECTROSCOPIC PROPERTIES

4.2.3.1 Visible/Ultraviolet Electron Absorption Spectrum

The electron absorption spectrum of the chlorophylls and their derivatives is very characteristic and is attributed to the system of conjugated double bonds making up the basic porphyrin structure. Electron transitions in the chlorophyll molecules, detected by photosensitive optical equipment, produce absorption bands (Clydesdale and Francis, 1976; Holden, 1976; Jackson, 1976). The intensity of a particular absorption band is normally expressed by its *molar specific coefficient of extinction* (ϵ), which according to the Beer-Lambert law depends on the optic density (A), the molar concentration (C), and the optical pathway of the light beam through the solution (l) [expressed in centimeters].

The porphyrins and related compounds have long been of interest because of the sharpness of the absorption bands in the visible and ultraviolet regions. This feature has been very useful in supplying information about both the overall structure of the macrocycle and the nature of the groups included in it.

The absorption spectra of all the porphyrin pigments are characterized by a number of relatively pronounced bands in the yellow, red, and near-infrared regions and a band of strong absorption in the violet or near-violet region, denominated the "Soret band." The presence of the latter indicates that there has been no cleavage of the basic porphyrin structure (Schwartz et al., 1957).

The dielectric properties of the solvent also affect the spectrum. The spectral bands of chlorophylls in ether solution are very sharp because of the weak interaction between this solvent and the pigment molecules. In solvents that are more polar, such as acetone, methanol, ethanol, or mixtures of these with water, the interaction with the pigment causes displacement of the absorption maxima toward higher wavelengths, while the intensity (peak height) and specificity (peak width) of the absorption band decrease.

Both chlorophyll *a* and chlorophyll *b* present absorption maxima in the blue-violet region, with characteristic peaks at 428 and 454 nm and smaller ones at 410 and 430 nm, respectively. In addition, there are four pronounced bands between 500 and 700 nm in the red region. This variance is the result of the small structural difference between the two chlorophylls. The displacement toward the green region of the spectrum changes the greenish-blue color of chlorophyll *a* to yellowish-green in chlorophyll *b*.

The substitution of Mg by hydrogens in chlorophyll *a* produces a marked hypsochromic displacement in the Soret band, from 428 to 408 nm, whereas the displacement in the secondary band (Q band) is bathochromic and less marked, from 662 to 666 nm, with an increase in the peak ratio (Figure 4.5). In the case of chlorophyll *b*, there are similar changes in the absorption spectrum with the pheophytinization reaction. The Soret band is displaced from 454 to 430 nm, and the secondary band is displaced from 646 to 656 nm (Figure 4.6). Insertion of other divalent cations such as Cu or Zn modifies the absorption spectrum in the opposite sense. The shape of the spectrum, the location of the absorption maxima, and the peak relation are very similar to those of the spectrum of the original complex with

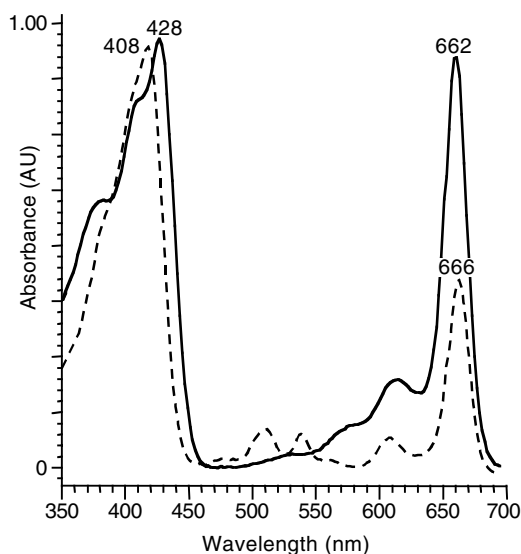


FIGURE 4.5 Electronic absorption spectra of chlorophyll *a* (—) and pheophytin *a* (- - -).

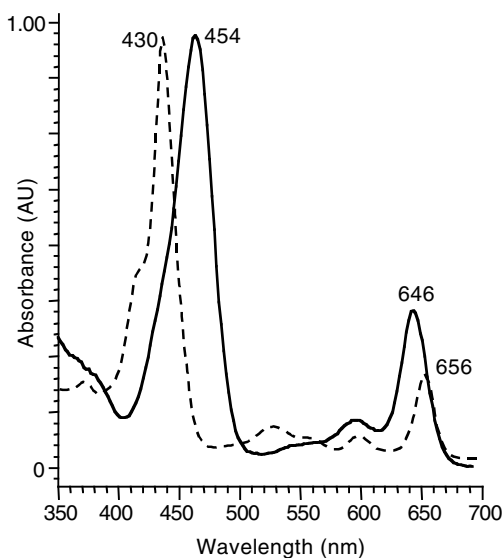


FIGURE 4.6 Electronic absorption spectra of chlorophyll *b* (—) and pheophytin *b* (- - -).

Mg, although as they are not identical, they can be readily distinguished. As an example, Figure 4.7 shows the electron absorption spectra of Cu-pheophytin *a* and pheophytin *a*. The most conspicuous changes in the absorption spectrum are caused by structural modifications affecting the chelate, which alter the chromophore properties.

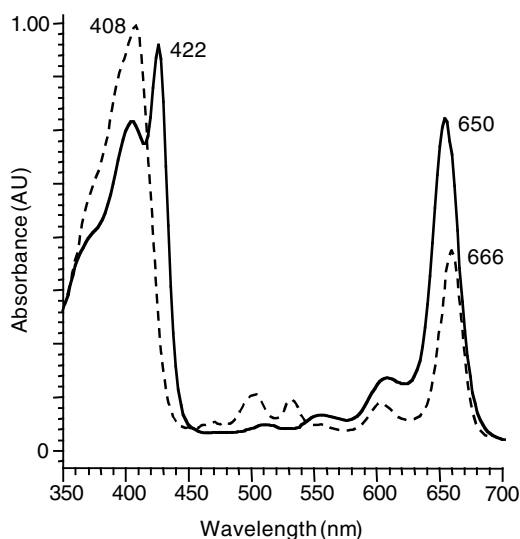


FIGURE 4.7 Electronic absorption spectra of pheophytin *a* (---) and Cu-pheophytin *a* (—).

In contrast, de-esterification of phytol in the chlorophyll molecule does not affect the chromophore structure, so the electron absorption spectrum is unaltered. Thus, chlorophyllides and pheophorbides have the same spectroscopic properties as their respective precursors, chlorophylls and pheophytins. The same is true of some of the reactions that affect the isocyclic ring, such as epimerization and decarbomethoxylation on C-13².

Primary allomerization reactions that give rise to hydroxy- or methoxy-chlorophylls also do not affect the spectroscopic properties. In contrast, other oxidative reactions involving opening of the isocyclic ring or the formation of a lactone ring do cause important changes in the electron absorption spectrum.

Figure 4.8 shows the specific differences between the spectrum of chlorophyll *a* and that of Mg-phytyl-chlorin *e*₆, the product of solvolysis of the isocyclic ring of chlorophyll *a*. There is a hypsochromic displacement of 14 nm in the Soret band and of 16 nm in the maximum of the red region. A similar change takes place in the corresponding derivative of series *b* (Figure 4.9). Figure 4.10 shows the change in the absorption spectrum of pheophytin *a* caused by solvolysis of the isocyclic ring. There is a hypsochromic displacement of some 8 nm in the Soret band and another, much less marked, displacement in the red band (4 nm), with considerable changes in the shape of the spectrum.

The spectral changes caused by the inclusion of a lactone ring in the structure of pheophytin *a* can be observed in Figure 4.11. The spectrum of 15¹-OH-lactone pheophytin *a* shows a 10-nm hypsochromic displacement of the Soret band. In the case of phytol-purpurin 18*a*, the most striking displacement is that of the maximum of the red region, a bathochromic change of around 30 nm.

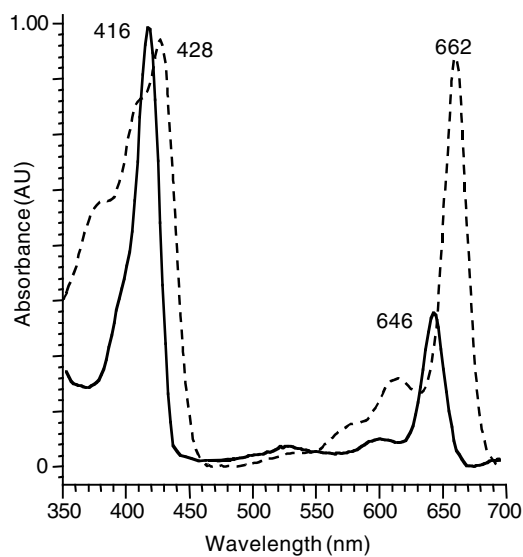


FIGURE 4.8 Electronic absorption spectra of chlorophyll *a* (---) and Mg-phytol-chlorin *e*₆ (—).

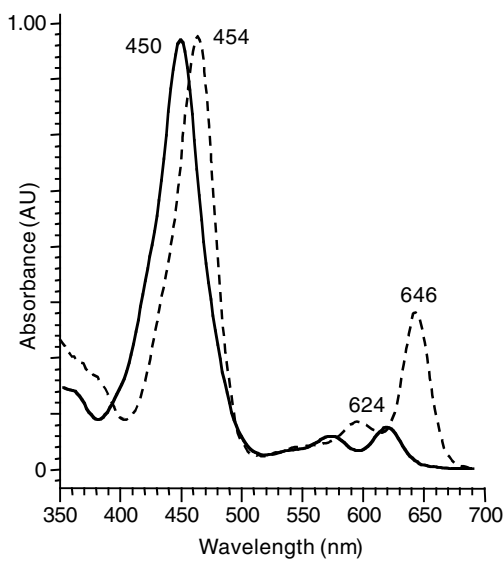


FIGURE 4.9 Electronic absorption spectra of chlorophyll *b* (---) and Mg-phytol-rhodin *g*₇ (—).

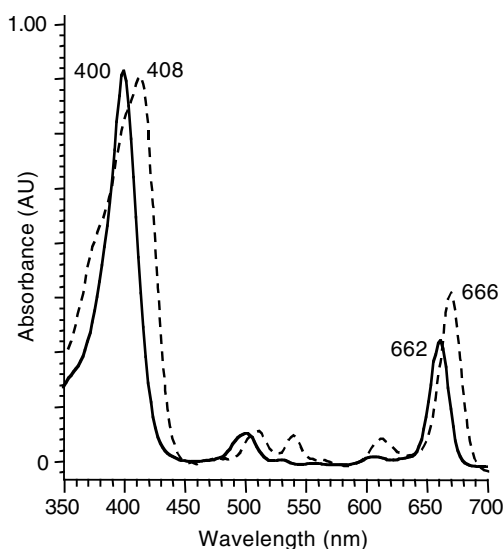


FIGURE 4.10 Electronic absorption spectra of pheophytin *a* (---) and phytol-chlorin *e*₆ (—).

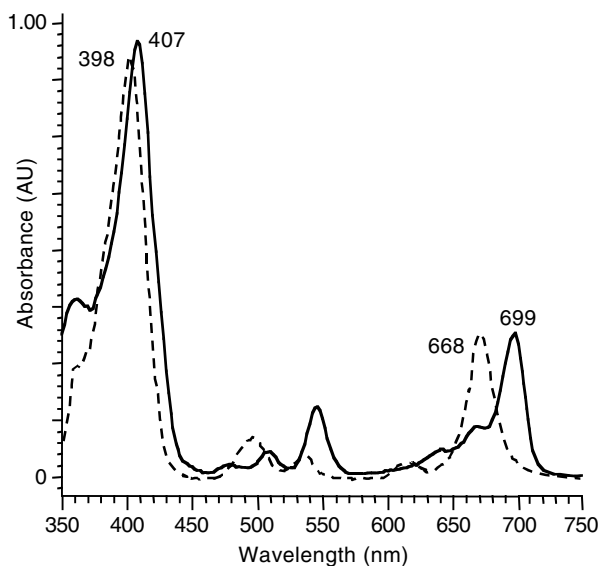


FIGURE 4.11 Electronic absorption spectra of 15^l-OH-lactone pheophytin *a* (---) and phytol-purpurin 18 *a* (—).

Finally, chlorophyll derivatives in which there has been a cleavage of the porphyrin macrocycle show no electron absorption band in the visible region and are therefore colorless compounds.

4.2.3.2 Fluorescence Spectrum

The fluorescence spectra of chlorophylls are similar to those of visible absorption. That of chlorophyll *a* shows a higher sensitivity at maximum and minimum wavelengths than that of chlorophyll *b*. The spectra of pheophytin *a* and pheophytin *b* are similar to those of the corresponding chlorophylls (Goedheer, 1966). The fluorescence spectrum of the chlorophylls is affected by solvents, with fluorescence being lost in pure and dry hydrocarbons (Livingston et al., 1949). It is also affected by temperature, molar concentration, and the degree of solvation (Kirk and Tilney-Basset, 1978).

Fluorimetric methods have not been used as widely as ultraviolet-visible (UV-Vis) spectrophotometric ones, although they are much more sensitive: detection requires only 1% of the amount of pigment necessary in the latter method (Virgin, 1955, 1961). With this technique, chlorophylls and/or their derivatives have been detected quantitatively in picomole amounts (Bazzaz and Rebeiz, 1979; Falkowski and Sucher, 1981; Shioi et al., 1983; Zonneveld et al., 1984). Very low amounts of chlorophylls *a* and *b* can also be determined from their mixtures, as the fluorescence of one is independent of that of the other (Goodwin, 1947).

Although UV-Vis spectroscopy has been more widely used than fluorescence, the latter is becoming the preferred technique for studies involving chlorophylls in photosynthesis. Different systems of equations have been developed for the fluorimetric determination of the concentration of chlorophylls *a* and *b* or more complex mixtures that also include pheophytins, chlorophyllides, and pheophorbides (Boardman and Thorne, 1971; Gross, 1987; White et al., 1972). Fluorescence is measured in the Soret band, where the differences for the various components are greater.

These compounds show a characteristic strawberry fluorescence under UV_{254 nm} light, a property used to detect chlorophylls and pheophytins on thin-layer chromatography (TLC) plates (Zonneveld et al., 1984). Although this property is very generalized in most chlorophyll derivatives, there are some in which it is absent, making it a very useful test of identification. This is the case of the metallochlorophylls of Cu (Mínguez-Mosquera et al., 1996) and the purpurins 18 (Mínguez-Mosquera et al., 1993). Fluorimetric detectors are also used as selective monitors coupled with high-performance liquid chromatographs (HPLC) (Endo et al., 1992; Mantoura and Llewellyn, 1983; Shioi et al., 1983).

4.2.3.3 Other Spectroscopic Properties

Nuclear magnetic resonance (NMR), MS, and infrared (IR) spectroscopy have been used successfully in the structural elucidation of chlorophylls and derivatives (Jackson, 1976; Janson and Katz, 1978; Katz et al., 1966; Scheer and Katz, 1975). Studies of hydroporphyrins and chlorophylls also have been carried out with Raman resonance spectroscopy (Schick and Bocian, 1987).

Specific functional groups have been identified by IR spectroscopy (Katz et al., 1963). The IR spectrum of chlorophylls and their derivatives has been determined

in emulsions in mineral oil, in potassium bromide disks, and in solution. The ceric properties of chlorophylls hinder the preparation of emulsions and disks; moreover, the spectrum is resolved better in solid state than in solution (Schwartz et al., 1957). The presence of functional groups on the side chain of chlorophylls increases the IR absorption bands in the expected regions of the spectrum. The spectra of chlorophylls *a* and *b* in nonpolar solvents, such as carbon tetrachloride, are surprisingly similar, considering that the two molecules are different, in that on C-7, chlorophyll *b* has a formyl group instead of a methyl group. In contrast, in more polar solvents, such as tetrahydrofuran, the two chlorophylls have very different spectra. This phenomenon is particularly marked in the carbonyl region. It has been known for some time that the spectrum of these compounds in nonpolar solvents depends on their concentration.

The first authors to obtain complete structural information using NMR were Closs et al. in 1963. ^1H -NMR has been the most convincing method thanks to the resonances of the protons in the porphyrin ring structure. A factor in the success of the technique is that it requires only a small amount of pure pigment (100 μg) (Bazzac and Rebeiz, 1979; Scheer, 1988), compared with ^{13}C -NMR (Katz, 1972), which requires a high concentration of pigment.

It was not possible to use MS for the characterization and identification of chlorophylls and derivatives until the development of desorption methods (desorption ionization) appropriate for nonvolatile and thermolabile compounds. The mass spectrum of chlorophylls has been obtained using "laser desorption" (Grote Meyer et al., 1986; Posthumus et al., 1978; Tabet et al., 1985), field desorption (Dougherty et al., 1980), plasma desorption (Chait and Field, 1984; Hunt et al., 1981), fast atom bombardment (FAB) (Baber et al., 1982; Brereton et al., 1983; Hyvarinen and Hyninen, 1999; Mínguez-Mosquera et al., 1996, Teng and Chen, 1999), "in-beam" electron ionization (Constantin et al., 1981), and "electrospray ionization" (EIS) (Gandul-Rojas et al., 1999a). A combination of the techniques of desorption and "tandem mass spectroscopy" (MS-MS) has also been used for the characterization of chlorophylls and derivatives (Bricker and Russell, 1986; Grese et al., 1990; Jackson, 1979; Van Breemen et al., 1991a). The latest research in this field coupled HPLC with MS, using as ionization source FAB (Grese and Gross, 1992; Kostiainen et al., 1995; Van Breemen et al., 1991b) or chemical ionization at atmospheric pressure (APCI) (Eckardt et al., 1991; Harris et al., 1995; Lee et al., 1998; Verze gnassi et al., 1999; Zissis et al., 1999).

4.2.4 CHLOROPHYLL STABILITY DURING FOOD PROCESSING

Chlorophylls form part of the natural human diet as integral components of edible plants. However, once the plants have been harvested, the chlorophylls begin to degrade and can become transformed into other colored derivatives or can be degraded totally to colorless compounds if the material is stored for a long period. Vegetable processing, particularly by heating, also leads to transformation of the chlorophylls into their derivatives.

To a greater or lesser extent, all processing systems cause changes in the chloroplast pigments present and, thereby, in the apparent color of the food. Numerous studies have been carried out in an attempt to prevent these changes and to preserve the bright green color that makes green vegetables look appetizingly fresh. Nevertheless, there is no evidence demonstrating that the structural changes occurring in chlorophylls during food processing cause deterioration in their functional properties. On the contrary, the derivatives formed, mainly pheophytins, can preserve or even strengthen such properties (Chernomorsky et al., 1999; Higashi-Okai et al., 1998; Yang et al., 1999). In contrast, chlorophyll transformation reactions that lead to colorless products play a very negative role in color and in functional activity of the food, since the cleavage of the porphyrin macrocycle presumably suppresses the capacity of these compounds to bond with DNA and mutagenic agents (Dashwood, 1997).

The whole range of chlorophyllic pigments can be found in processed vegetables, to a greater or lesser extent depending on the characteristics of the plant material and the physicochemical conditions under which the process takes place. Factors such as temperature, pH, enzyme action, oxygen, light, and storage time determine the transformation of these compounds (Gross, 1991).

Although the natural concentration of chlorophyll *a* is higher than that of chlorophyll *b* in all green tissues, their transformation products do not follow the same pattern, as the reaction rate is not the same in the two chlorophylls. The many kinetic studies of chlorophyll transformation reactions all agree that those involving the chelate, such as pheophytinization or the insertion of divalent cations, are more rapid in the *a*-series compounds (Lajollo et al., 1971; Mackinney and Joslyn, 1940; Nonomura et al., 1994; Schwartz and Lorenzo, 1989). In contrast, the enzymatic de-esterification of phytol or the formation of pyroderivatives is more rapid in the *b*-series compounds (Mínguez-Mosquera et al., 1994a, 1994b; Schwartz and von Elbe, 1983).

The purpose of conservation treatments is to conserve the properties of the material as unaltered as possible, destroying microorganisms that might alter the food or cause disease under certain storage conditions. Necessarily, the different technological alternatives for the conservation of foods can have different effects on the type and proportion of chlorophyll derivatives formed. Table 4.1 summarizes the pigments that may be present in plant foods, depending on the type of processing to which they have been subjected.

Postharvest conservation of vegetables normally results in a loss of quality, caused by water loss, biological oxidation or other chemical changes, and physiological ruptures. The generalized liberation of acids following breakdown of cell integrity is very common in plants, in which the pH of the internal medium can reach 5.5 units. The most common reaction under such conditions is pheophytinization, since the central atom of Mg of the chlorophyll molecule is readily liberated under slightly acid conditions and replaced by hydrogen. The rate of chlorophyll conversion to pheophytin will differ with the degree of acidity of the food. The breakdown of cell integrity allows contact of endogenous enzymatic systems with their substrates, giving rise to hydrolytic and/or oxidative reactions in the chlorophylls. In

TABLE 4.1
Profile of Chlorophyll Pigments in Vegetable Products as a Function of the Technological Process Applied

Pigment	Green Vegetable	Green Vegetable after Treatment ^a			
		Freezing	Blanching	Sterilization	Fermentation
Chlorophylls					
Chlorophyll <i>a</i>	++++	++++	+++	—	—
Chlorophyll <i>b</i>	++	++	++	—	—
Mg-free derivatives					
Pheophytin <i>a</i>	—	tr	+	++	+++
Pheophytin <i>b</i>	—	tr	tr	+	++
Phytol-free derivatives					
Chlorophyllide <i>a</i>	—	—	*	—	—
Chlorophyllide <i>b</i>	—	—	*	—	—
Mg-phytol-free derivatives					
Pheophorbide <i>a</i>	—	—	*	**	**
Pheophorbide <i>b</i>	—	—	*	*	*
Oxidized derivatives					
Pyroderivatives ^b	—	—	—	***	tr
Isomers					
Epimers C ₁₃ ^c	—	tr	+	+	+

^a Codes. The number of symbols indicate relative abundance: +, pigments generally presents in fresh or processed green vegetables; *, pigments present in vegetables with chlorophyllase activity; —, pigment absent; and tr, pigment in amount of traces.

^b Include the pyroderivatives of all the chlorophyll pigments.

^c Include the epimers of all the chlorophyll pigments.

the case of materials with chlorophyllase activity, the chlorophylls will be converted partially to chlorophyllides, in which the Mg can also be substituted by hydrogens, with transformation to pheophorbides. On the other hand, oxidative reactions catalyzed by lipoxygenase, peroxidase, or chlorophyll-oxidase lead to chlorophyll degradation to colorless products. Storage in a controlled atmosphere, combined with refrigeration, slows such reactions (Gross, 1991; Perrin and Gaye, 1986; Shewfelt et al., 1983; Stewart and Uota, 1971; Wang et al., 1971).

Blanching is one of the most common initial steps in the preparation of frozen, canned, or dehydrated vegetables to preserve the natural green color and thereby the quality. It is a mild heat treatment — not exceeding a temperature of 100°C — normally carried out with steam or boiling water. Modern methods also use micro-ovens or convection ovens. However, it is not possible to establish a norm for the blanching method that achieves the greatest retention of chlorophylls. The process inactivates the enzymes present, shrinks the material, expels gases from the tissues, reduces any initial infection, and prevents the formation of off-flavors. The chlorophyll derivatives commonly found in blanched vegetables are pheophytins, chlorophyllides, pheophorbides, and the respective C-13² epimers (Dietrich and

Neumann, 1965; Drake et al., 1981; Muftugil, 1986; Sistrunk et al., 1977; Teng and Chen, 1999).

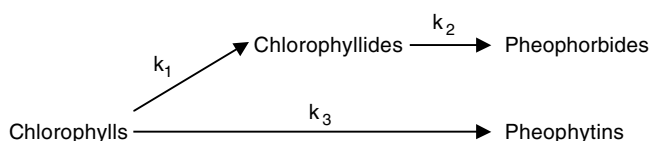
The extent of the reaction converting chlorophylls into pheophytins is directly related to the temperature and the time that the blanching treatment lasts (Walker, 1964). Blanching at low temperatures (65 to 80°C) favors activation of the enzyme chlorophyllase and thus the formation of dephytylated chlorophyll derivatives (chlorophyllides and pheophorbides). At higher temperatures (around 100°C), the enzyme is partially inactivated, but favors the parallel reaction of pheophytinization. The extent of chlorophyllide and pheophorbide formation will depend not only on the specific conditions of the technological treatment, but also on the level of chlorophyllase activity in the plant material (Jones et al., 1963).

Another type of food processing that involves mild heat treatment is dehydration. This method of conservation reduces water activity by removing water from the product by evaporation or lyophilization. The conversion of chlorophyll *a* into pheophytin *a* takes place even at very low concentrations of water (Lajollo and Lanfer Marquez, 1982), but can be retarded by reducing the moisture content of the vegetable or decreasing its activity. As in blanching, the temperature used for food dehydration determines the percentage of pheophytin formation; greater amounts of this pigment are found when samples are heated to higher temperatures (Berset and Caniaux, 1983; Gross, 1991).

During the process of sterilization by heating, which involves a stronger heat treatment (to temperatures above 100°C), the vegetables undergo a marked color change from bluish-green to olive green by the generalized conversion of chlorophylls to Mg-free derivatives. Sterilization is a more drastic process for the chlorophylls, in which, besides the chelate reaction, there is also oxidation on the isocyclic ring, with complete transformation to pheophytins and pyropheophytins. The degradation mechanisms suggested include two reactions in series: (1) pheophytinization and (2) pyropheophytinization (Schwartz et al., 1981).

The extent of pyropheophytin formation is considered to be related with the severity of the heat treatment. In processes setting up convection currents, the heating rate is higher and the treatment time is lower than when heat is transmitted by conduction. The percentage of pyroderivatives formed is thus higher in the latter. In the system known as high temperature and short time (HTST), which achieves sterilization using high temperatures (150°C) and short times, the percentage of these derivatives formed is lower (Schwartz and Lorenzo, 1989). This system improves product quality, but may leave a certain residual enzyme activity that causes deterioration in color and flavor during storage (Clydesdale et al., 1972).

In vegetable foods subjected to lactic acid fermentation, activity of the enzyme chlorophyllase is very often favored during the process, and dephytylated chlorophyll derivatives are found (Blanc, 1973; Jones et al., 1963; Mínguez-Mosquera et al., 1989a; Ruskov and Malchev, 1971; White et al., 1963). The degradation mechanism proposed is as follows:



While the enzyme chlorophyllase is active, some of the chlorophylls give rise to chlorophyllides. At this stage, the remaining chlorophylls have not been affected by the action of the enzyme and are a substrate available for the subsequent pheophytinization reaction. This takes place in parallel with the formation of pheophorbides (Mínguez-Mosquera et al., 1994a). Pyropheophytins are also detected in fermented vegetables, with their presence being attributed to the heat generated in the process (Mínguez-Mosquera and Gallardo-Guerrero, 1995; Schwartz et al., 1983; Takeda et al., 1990).

During food conservation, chlorophyll degradation may reach the stage of oxidation to colorless products. One of the oxidative enzymes apparently involved in the decoloration of vegetables is lipoxxygenase, which catalyzes the coupled oxidation of polyunsaturated lipids and pigments (Holden, 1965; Mínguez-Mosquera, 1990; Walker, 1964). The free radicals formed as intermediate products in the catalysis of peroxidation are directly responsible for the cooxidation of chlorophylls. The heat treatment of blanching inhibits lipoxxygenase activity and prevents development of these oxidative reactions during the conservation of frozen vegetables (Buckle and Edwards, 1970). Heat and light also have a destructive effect on chlorophyll (Walker, 1964).

In vegetable oils, the most common reaction is again pheophytinization as a result of the generalized liberation of acids produced by mechanical breakdown of the plant tissue. Pheophytin *a* is the major pigment in this product, although slight differences are found depending on whether the oil has been extracted by physical procedures, as in the case of olive oil (Mínguez-Mosquera et al., 1990; Mínguez-Mosquera et al., 1992a; Rahmani and Csallany, 1991), or chemical ones using solvents (Usuki et al., 1984; Ward et al., 1994). The conditions during physic extraction processes favor activation of the enzyme chlorophyllase, and dephytylated chlorophyll derivatives, mainly pheophorbide *a*, can be found in the oil (Gandul-Rojas and Mínguez-Mosquera, 1996 a, b). During oil storage, oxidative reactions also take place, and pyropheophytins and allomerized chlorophyll derivatives are found (Gandul-Rojas et al., 1999b; Ward et al., 1994). During the refining of edible oils from oil seeds, the chlorophyll compounds are removed almost entirely by processes of adsorption on decoloring clays (bleaching) and/or treatment at high temperature (deodorization) (Usuki et al., 1984).

4.2.5 CHLOROPHYLLS AS ADDITIVES

Of all the possible transformations of the chlorophyll molecule during food processing and conservation, those affecting the chelate cause the most striking changes in the color of the product. The substitution of Mg by hydrogens in the porphyrin ring means a considerable reduction in quality, as it causes a dramatic color change from bright green to olive green and the disappearance of the attractive aspect of a fresh vegetable. Chlorophylls, chlorophyllides, or any other product of chlorophyll oxidation are susceptible to this transformation whenever the essential condition of acidity is present.

Numerous studies have been carried out to prevent this degradation, although none has achieved an optimum result. They include the control of pH with alkalinizing

substances (Clydesdale and Francis, 1968; Eheart and Odland, 1973); favoring of chlorophyllase action to form chlorophyllides, which are more thermostable than chlorophylls (Clydesdale and Francis, 1968); or heat treatment at HTST (Tan and Francis, 1962). Most of the methods tested were able to retain more chlorophylls, but this effect did not last during prolonged storage.

The only alternative for obtaining processed foods with a stable green color is the addition of a colorant. This is normally used to strengthen the green color of a vegetable or to supply green coloration to a food that does not possess color or has lost it during processing. Examples of such foods are confectionery, desserts, snacks, and drinks, in which colorants are also used to help the consumer to identify the product or simply to give it a more attractive appearance.

Chlorophyll itself is not particularly appropriate as an additive; it is not only unstable, but is also insoluble in water. The stability of the molecule can be enhanced by substitution of the Mg ion in the chlorophyll molecule by other divalent cations such as copper or zinc, makes the complex less liable to modification of the chelate under acid conditions, and conserves a stable green color (Humphrey, 1980). The first observations in this sense referred to the regreening of canned vegetables during storage, which was attributed to the limited formation of metallochlorophyll complexes of Cu and Zn (Fishbach, 1943; Schanderl et al., 1965). As a result of these observations, the Veri-Green® process was patented in 1984 (Segner et al., 1984), whereby Zn ions are incorporated into the lining of the metal containers, stabilizing the color during blanching by the formation of Zn complexes of pheophytin and pyropheophytin (von Elbe et al., 1986). Veri-Green® products have not been successful in the United States because the amount of Zn required to achieve an optimum color exceeds 75 ppm, the upper limit admitted in the United States for this metal. In Europe, there are no set limits. Subsequent patents add solutions of Cu or Zn salts during food processing (Canjura et al., 1999; Laborde and von Elbe, 1996; Leake and Kirk, 1992), and very recently, improvement of the color in green vegetables has been achieved using Zn salts coupled with continuous flow aseptic processing technology (Canjura et al., 1999).

As a result of the capacity of these chlorophyll metal complexes to preserve the green color, the Cu complex of a water-soluble chlorophyll derivative obtained by alkaline treatment was marketed for the first time in 1984. This chlorophyll derivative, known as chlorophyllin, is the result of chemical hydrolysis of the alcohol phytol, of the methyl esters, and of the cyclic β -ketoester (ring V) in the chlorophyll structure. The food colorant, known as sodium-copper (Na-Cu) chlorophyllin (E-141 ii) is the Na salt of the chlorophyll saponification product in which the Mg^{2+} has been replaced by Cu^{2+} . The commercial product is a mixture of different Cu-chlorophyll derivative complexes that can include Cu-pheophorbide *a*, Cu-chlorin *e*₆, Cu-chlorin *e*₄, Cu-rhodin *g*₇, and other degradation products (Chernomorsky et al., 1997; Inoue et al., 1994; Yasuda et al., 1995). This product is bluish-green in color, is moderately soluble in water, and resists the heating conditions used during canning.

For liposoluble foods, there is colorant E-141 i, known as "copper complexes of chlorophylls." It is obtained by addition of a Cu salt to the solvent-extracted product from an edible plant source. Its main coloring material is Cu-pheophytin,

although other compounds are also present, such as carotenoids and lipids from the raw material.

Currently, Na-Cu chlorophyllin is the most widely used natural green colorant. This chlorophyll derivative has been the basis of many of the studies carried out on the functional value of chlorophyll compounds, and important antimutagenic and anticarcinogenic properties have been found. Research has been conducted into the capacity of chlorophyllin to protect DNA against ionizing radiation and its considerable potential for prevention of mutation-based health impairment, including cancer and other degenerative diseases (Kumar et al., 1999). Other studies seem to demonstrate that chlorophyllin is effective as an antimutagen against the genotoxic side effects that antitumoral agents can have on healthy cells (Gentile et al., 1998; Te et al., 1997).

4.3 ANALYSIS OF CHLOROPHYLLS

4.3.1 EXTRACTION TECHNIQUES

For the extraction of chlorophylls from plant tissues, the AOAC (1984) recommends an initial step of milling or blending (homogenization) of the tissue, which can be improved by the addition of quartz sand to the mortar. Pigment extraction is normally carried out by adding small amounts of solvent to the triturate. Solvent and plant material are usually left in contact for a time to assist the extraction by maceration, but precautions are necessary because the chlorophylls can be easily degraded to other products. The solid material is removed by filtration through paper, glass wool, inert diatomaceous clay, or by centrifugation, although filtration is preferred for its better guarantee of completely recovering the extract without solid residues. The extraction procedure is repeated until there are no traces of pigment in the sample. Finally, the filtrates are combined and taken to volume. All these operations are carried out rapidly, in darkness or under weak green light, to prevent photodegradation and/or allomerization of the pigments.

Although the extraction process is simple, each plant product requires an appropriate method, and the need often arises to vary the methodology, adapting it to the particular case. Many methods are described in the References — some quantitative, others qualitative, while others are semi-quantitative — to achieve an adequate degree of exhaustion in the extraction using a simple, rapid method. The techniques are varied — percolation, maceration, sonication, diacolation, etc. — and are generally useful with almost any inert organic solvent, although most use methanol or acetone because they are extraction solvents of medium-high polarity, little selectivity, and a wide range of solubilization (AOAC, 1984; Bacon and Holden, 1967; Brunisma, 1963; Buckle and Edwards, 1970; Clydesdale et al., 1970; Holden, 1976; Humphrey, 1980; Mínguez-Mosquera and Garrido-Fernández, 1985; Mínguez-Mosquera, 1991a, b; Mínguez-Mosquera et al., 1992a; Moran and Porath, 1980; O'Neill and Crener, 1980; Oquist and Samuelsson, 1980; Schwartz et al., 1981; Strain and Svec, 1966; Strain, 1954; Strain et al., 1971; Svec, 1978; Sweeney and Martin, 1958; Van Buren, 1985).

Although the extraction stage is largely mechanical, the operation must be correctly performed to guarantee the consistency of the subsequent analysis. It is essential that the pigment extraction system chosen is complete and reproducible and that there is no formation of degradation compounds during the process. Chlorophyll pigments are, by nature, extraordinarily labile. The wrong choice of extraction system or inappropriate analytical conditions (excessive light, high temperature, long extraction time, etc.) may mean epimerization on C-13² of the isocyclic ring or even the formation of pheophytins by loss of the central atom of Mg. In solvents with exchangeable protons, for instance methanol or ethanol, epimerization is favored, so if they are used as extractants, the operation should be carried out as quickly as possible. Furthermore, if the buffer capacity of the solvent is low, the liberation of acids from the tissue could increase the rate of pheophytinization. To avoid such a possibility, the buffer capacity of the extractant is normally increased by the addition of basic salts (AOAC, 1984). In tissues with high chlorophyllase activity, enzymatic de-esterification of chlorophylls and pheophytins can take place during the extraction phase.

All these products are formed under relatively mild conditions, so manipulation of samples containing chlorophyll compounds must be carried out at temperatures below 40°C, under weak or green light, preferably with inert atmosphere, and using solvents that do not allow alterations of the natural compounds. All the circumstances mentioned previously mean that work with chloroplast pigments must be performed with the greatest care, as they can rapidly be altered, forming artifacts.

For the extraction of pigments in foods with scant lipid content, any organic solvent can generally be used, since all the pigments will be soluble to a greater or lesser extent, and the exhaustion of color with extraction is only a question of repeating the process. When the aim is not very specific, acetone achieves extraction in a reasonable time and with little expenditure of solvent.

The end of the process is normally signaled by the obtaining of colorless extracts. Theoretically, this point should in every case yield extracts with identical pigment content, both qualitatively and quantitatively. In practice, it does not normally happen. For solvents with a high-penetration capacity, extraction is usually easy, with no need to triturate the sample exhaustively. For solvents that are desiccant (acetone or ethanol) or hydrophobic (hexane), access to the internal structures containing the pigments is hindered by dehydration of the sample and subsequent compacting or by repulsion of the solvent. This drawback is avoided using an adequate and repeated trituration.

A particular case is the study of samples with a high content of water. Its presence in the extract may be due to the tissue's having either high moisture or little pigmentation, so a substantial weight of starting sample will be necessary. In either case, the contribution of water will be considerable and will impede the evaporation of solvent. One simple, useful solution is to transfer the acetone extract to ethyl ether.

Another type of sample requiring specific treatment is that with a high lipid content. The presence of such compounds interferes in the whole process of isolation and subsequent purification. They can be removed by means of selective phase distribution, using two solvents — one with high affinity for fat and the other with selective pigment-retention capacity.

4.3.1.1 Methodology

4.3.1.1.1 Simple Material

The sample (0.5 to 10 g, depending on the pigment content) is triturated for 1 min with 50 ml of acetone saturated with MgCO_3 to minimize pheophytinization during maceration. The mixture is filtered, and the solid residue is collected and treated repeatedly with acetone until the extract is colorless. The acetone extracts are combined, and the volume is reduced to dryness in a rotavapor at a temperature below 30°C.

In the case of samples with high-moisture content, the final volume of acetone extract is mixed with ether to form a single phase. The solution is separated by adding aqueous solution of sodium chloride (NaCl) (10%) and the ether phase is washed repeatedly. Reextraction with ether is continued until the aqueous phase is completely colorless. If there are no highly polar pigments present, the first transfer normally contains all the pigmentation. The ether phase is finally filtered through a bed of anhydrous Na_2SO_4 to remove the water completely and is taken to dryness in a rotavapor.

4.3.1.1.2 Complex Material (High Lipid Content)

Between 5 and 10 g of sample are weighed and transferred quickly to a precipitating flask containing some 50 ml of *N,N*-dimethylformamide (*N,N*-DMF) saturated with MgCO_3 . The mixture is triturated for 1 min, and the homogenate is filtered *in vacuo* through coarse filter paper. The solid residue is collected, and the procedure described above is performed until the filtrate is colorless. Normally, four extractions are sufficient.

The filtrates are combined in a 500-ml decanting funnel and treated with 70 ml of hexane to extract the fatty matter. The mixture is shaken for 1 min and allowed to stand until complete separation of phases. The upper layer, slightly yellow, retains lipids, carotenes, and di-esterified xanthophylls. The *N,N*-DMF hypophase, containing the rest of the pigments in solution, is treated twice more with hexane to remove completely any lipid remains. The three phases of hexane are combined and washed with 50 ml of *N,N*-DMF.

The *N,N*-DMF solution is then transferred to a 1000-ml decanting funnel containing 400 ml of 10% NaCl solution at a temperature close to 0°C. Hexane (70 ml) is added together with 70 ml of ethyl ether, shaken, and then allowed to stand until complete separation of phases (30 min). The aqueous phase is again treated with ethyl ether (50 ml). The remaining water-soluble compounds are retained in the aqueous phase, which is discarded. The organic phases are combined and washed several times with an aqueous solution of Na_2SO_4 (2%) and then filtered through a bed of anhydrous Na_2SO_4 . The water-free solution is concentrated in a rotavapor to dryness at a temperature below 30°C (Mínguez-Mosquera and Garrido-Fernández, 1989).

4.3.2 CHROMATOGRAPHIC SEPARATION TECHNIQUES

The analysis of chlorophylls must be completed as soon as possible after extraction, as artifacts can be formed during storage of the samples (Braumann and Grimme,

1979). It is essential that storage is under N₂ atmosphere and at −30°C. The precautions already mentioned for the manipulation of samples during extraction must be continued during separation. It is also important that the storage conditions are appropriate for the chromatographic supports, as indicated by the manufacturer (Shioi, 1991).

4.3.2.1 Column Chromatography

The classic chromatography experiments carried out by Tswett (1906) on a column of calcium carbonate allowed the separation of chlorophylls *a* and *b* from the rest of the carotenoids and continues to be widely used today for their large-scale separation (1 to 10 mg). The adsorbents are chosen for its selectivity and nonreactivity with the pigments and/or solvents (Strain and Svec, 1969; Strain and Sherma, 1972). Some inorganic adsorbents alter the chlorophyll molecule, causing isomerization, and those having a slightly acid nature induce degradation to pheophytins (Bacon and Holden, 1967; Holden, 1976; Strain and Svec, 1969). Adsorbents used include starch, cellulose, certain simple sugars, polyethylene, silica, calcium hydroxide, calcium phosphate, and alumina (Anderson and Calvin, 1962; Bacon, 1965; De la Mar and Francis, 1969; Jeffrey, 1968; Jones, 1963; Perkins and Roberts, 1962; Smith and Benítez, 1955; Stobart et al., 1967; Sweeney and Martin, 1961; Tan and Francis, 1962). The ion-exchange and molecular sieve methods are commonly employed analytically, especially for the pretreatment of samples. DEAE-Sephacrose CL-6B chromatography is particularly useful for the separation (and removal) of carotenoids from plant extracts and for the separation of esterified and nonesterified chlorophylls and their derivatives. The chromatography should be carried out at low temperature and in a short time to prevent isomerization reactions (Omata and Murata, 1980, 1983).

4.3.2.2 Chromatography on Paper

For many years, this has been a simple technique widely used for the separation and analysis of chlorophylls. An outstanding advantage is its great versatility in mode of use (unidimensional, bidimensional, radial, radial with acceleration centrifugation, etc.). At the same time, it is proven that pigment recovery following this technique often exceeds that obtained with TLC. Nevertheless, the procedure is no longer used in analytical separations, as its resolution power has been surpassed by TLC or HPLC techniques. For more detail, the References include reviews of paper chromatography methods used for the recovery of chloroplast pigments, with information on the quality of the paper, solvent systems, and separation conditions (Šesták, 1958, 1980).

4.3.2.3 Thin Layer Chromatography

The low price and simplicity of this technique allow it to be used widely for the separation and monitoring of pigments. The choice of support is vital, as many authors have reported the formation of artifacts and/or decomposition products during TLC, caused by light, acidity of the silica, drying methods, oxidation, and/or

solvents. Given the lability of chlorophylls, recommended adsorbents are few: silica gel or other silicates; sugar (powdered sucrose); cellulose; polyethylene; and, more recently, “chemically bonded C₈ and C₁₈” silica gel, specifically for reversed-phase TLC. However, for qualitative identification, and with due precautions, it is an unsubstitutable technique (Bacon, 1966; Buckle and Rahman, 1979; Holden, 1976; Lord and Tirimanna, 1976; Mínguez-Mosquera et al., 1989b; Strain and Svec, 1969; Strain et al., 1967). It has been reported that saccharose plates produce fewer degradation products (Chan et al., 1970; Jeffrey, 1968; Sahlberg and Hynninen, 1984). Recently, the efficiency of this technique has been improved with the development of high-performance (HP) plates in both normal and reversed phases, which have been used for the general separation of chloroplast pigments (Mínguez-Mosquera and Gandul-Rojas, 1995; Mínguez-Mosquera et al., 1993; Sherma and Latta, 1978; Suzuki et al., 1987; Ziegler et al., 1988).

4.3.2.4 High-Performance Liquid Chromatography

The main advantage of this technique is a high resolving power of complex mixtures in a short time, together with the small sample size required. The time of analysis, including sample preparation, is normally less than with other chromatographic methods. In some cases, separation is completed between 15 and 30 min. Until now, no reference has reported sample degradation during the chromatographic analysis — an important criterion when choosing a particular analytical method. The interaction of chlorophyll pigments with light makes HPLC especially appropriate for their analysis, as spectrophotometry is normally used as a detection technique. These advantages, together with the high sensitivity of UV-Vis and fluorescence detectors, have enabled the quantitative analysis of individual chlorophylls in highly complex mixtures to be tackled successfully. This chromatographic technique, in the preparative version, is also particularly useful for collecting amounts of pure individual components for their identification (Eskins et al., 1977; Kuronen et al., 1993; Mínguez-Mosquera et al., 1996; Tonucci and von Elbe, 1992).

HPLC was first used to analyze chlorophyll pigments in 1975 (Evans et al., 1975). Since then, numerous works have applied it to the separation of carotenoids, chlorophylls, and chlorophyll derivatives in plant tissues and marine phytoplankton. Some methods have used normal-phase chromatography, mainly on silica gel columns, and others have used reversed-phase chromatography, generally on C₁₈ functionality columns. This type of column appears to have certain advantages over the former, avoiding problems of pigment degradation and long conditioning times (Mantoura and Llewellyn, 1983; Wright and Shearer, 1984).

Numerous works can be cited that use normal-phase silica columns and isocratic mixtures of solvents such as acetone–ligroin (20:80, v/v) for the quantification of chlorophylls and derivatives in phytoplankton (Jacobsen, 1978) or *iso*-octane–98% ethanol (9:1, v/v) in spinach (Stransky, 1978). Watanabe et al. (1984) separated chlorophylls and pheophytins using the isocratic mixture 2-propanol–*n*-hexane (3:97, v/v). This method yields chlorophylls with levels of purity above 99%. Abaychi and Riley

(1979), using the mixture petroleum ether–acetone–dimethylsulfoxide–diethylamine (75:23.25:1.5:0.25 v/v/v) as the mobile phase, detected and quantified 16 pigments of chlorophylls, derivatives, and carotenoids.

Gradient solvent systems generally improve the resolution of this chromatographic technique. Outstanding mixtures are isopropyl alcohol in hexane from 1 to 10% (Iriyama et al., 1978); *n*-heptane–ethyl ether–acetone (De Jong and Woodlief, 1978); and acetone in hexane at 8, 10, and 12% (Yoshiura et al., 1978).

Evans et al. (1975) were the first to use reversed-phase HPLC to separate these pigments, with a mixture of ethyl acetate in petroleum ether as the mobile phase. They achieved a good resolution of porphyrins obtained from different natural sources and of the derivatives pheophytins *a* and *b*. After collection of the pigment fractions, the mass desorption spectrum was obtained. The data of chromatographic retention and of the mass spectrum provided a complete characterization of the pigments. Subsequently, Eskins et al. (1977) developed a preparative method for the separation of chlorophylls and carotenoids in the diatom *Nitzschia closterium*, using a gradient system from 80% aqueous methanol to a methanol–ether mixture. A good resolution was obtained for chlorophylls *a*, *b*, and *c*; pheophytins; and carotenoids such as neofucoxanthin, diadinoxanthin, diatoxanthin, β -carotene, and others typical of plants.

Reversed-phase methods have generally used different mixtures of methanol–acetone (Schoch et al., 1978), methanol–water (Braumann and Grimme, 1979; Shoaf, 1978), or methanol–acetone–water (Rebeiz et al., 1978) in isocratic or gradient form. Other workers add to the methanol–water mixture a phase modifier in the form of a gradient that can be acetone (Mantoura and Llewellyn, 1983), ethyl acetate (Eskins and Dutton, 1979; Eskins and Harris, 1981; Schwartz et al., 1981; Schwartz, 1984), or tetrahydrofuran (Craft et al., 1992). Polyethylene powder has been used as the reversed phase in the separation of bacteriochlorophylls and can also be used for chlorophylls in plants (Chow et al., 1978).

The difficulty in separating acid chlorophylls has prompted considerable effort. To improve resolution of the dephytylated chlorophyll derivatives, ionic suppressing reagents have been added to the mobile phase. These include ammonium and tetrabutylammonium salts (Davies and Holdsworth, 1980; Mantoura and Llewellyn, 1983), acetic acid (Shioi et al., 1984; Tonucci and von Elbe, 1992), and 0.005 *M* NaCl (Suzuki et al., 1987). Mantoura and Llewellyn (1983) include the ionic reagent in both the sample and the mobile phase to obtain a good resolution of chlorophyllides and pheophorbides. This method enables qualitative and quantitative monitoring of numerous chlorophyll derivatives and carotenoids in acetone extracts of algae from both cultures and natural waters.

The modifications introduced by Mínguez-Mosquera et al. (1991b) to the eluent system described previously enable resolution of the chlorophylls and carotenoids present in olive fruits and of the degradation products originated during the fruits' lactic fermentation to prepare table olives. The same system is applied to the characterization of pigments of the algal flora growing under immobilization conditions in the treatment

of wastewaters (Mínguez-Mosquera et al., 1991c, 1992b). Subsequently, the resolution of mixtures of oxidized derivatives of pheophorbides, which include pyropheophorbides *a* and *b*, chlorin, rhodin, and purpurins *a* and *b*, has been achieved by introducing small modifications in the elution gradient (Mínguez-Mosquera et al., 1993).

At the same time, nonaqueous reversed-phase methods have been developed. Wright and Shearer (1984) used a linear gradient from 90% of acetonitrile to 100% of ethyl acetate to separate 44 pigments, including carotenes, xanthophylls, chlorophylls, and derivatives, in marine phytoplankton. Khachik et al. (1986) combined an isocratic elution and gradient of methanol, acetonitrile, methylene chloride, and *n*-hexane to separate the major constituents (xanthophylls, chlorophylls, and carotenes) in different vegetables.

All these methods, in normal or reversed phase, are currently used for HPLC separation of chloroplast pigments, with only slight modifications in the solvent systems and in the form of elution, depending on the raw material to be analyzed (Almela et al., 2000; Hyvarinen and Hynninen, 1999; Teng and Chen, 1999).

Detection is normally by absorption or fluorescence spectrophotometry. The high coefficients of extinction of the chlorophyll Soret band enable sensitive detection between 380 and 445 nm. This region of the spectrum also includes the carotenoids, which accompany the chlorophylls in plant pigment extracts and whose analysis and quantification are also usually of interest (see Chapter 3). When these compounds are not of interest, and their possible interference must be excluded, a selective detection of chlorophylls and derivatives can be carried out at 654 nm (Schwartz et al., 1981) or 667 nm (Schoch et al., 1978), where there is no absorption of these pigments. Detection of chlorophylls and derivatives by fluorescence emission has been proposed. This is much more sensitive than absorption and also avoids interference from the carotenoids. Normally, excitation is done in the Soret band absorption region, and the emission is measured between 650 and 670 nm (Brown et al., 1981; Shioi et al., 1984).

Although much research effort has been put into the development of modern techniques of HPLC, more is needed to refine the technique for analysis of complex samples of pigments. Advances are expected with the development of coupled HPLC-MS systems.

4.3.3 ISOLATION AND PURIFICATION

The pigments to be identified can be isolated and purified by preparative TLC. This technique isolates the highest number of pigments with an initial general chromatography, each one in sufficient amount to carry out the subsequent steps of purification prior to identification by specific tests.

Chromatographic development separates the pigments in inverse relation to their polarity. The hydrocarbons, which are the least polar pigments, advance with the solvent, leaving those of higher polarity retained close to the base. The separate bands in the resulting general chromatogram show the following characteristics: order and value of retention factor (R_f) indicating pigment polarity and color under white and ultraviolet (UV_{254,360}) light, enabling an initial assignation as chlorophyll

or carotenoid pigments. R_f values are defined as distance from origin to spot center/distance from origin to solvent front.

Each band is scraped from the plate and eluted with an appropriate solvent. In principle, acetone is recommended — it solubilizes all the pigments acceptably, and its low viscosity means that the adsorbent decants much quicker, so that well-clarified solutions are easily obtained. The pigment solution is filtered through a small amount of anhydrous Na_2SO_4 . The volume of solvent is reduced in a rotavapor, and the concentrated solution of pigment is purified by subsequent chromatographic developments.

Each band from the TLC plate is rechromatographed on the same support and with the same developer. After this initial purification, HPLC will show the number of pigments comprising each problem band. Normally, at this point, one will have some idea of the structure of such pigments to guide their separation by TLC, using different supports and developers depending on the case.

If there are no indications of the nature of the problem substances, the initial polarity shown by the compound in the general development can be used in choosing developers and supports to expand the region. The R_f value of the pigment in a TLC development is directly related to the polarity of the pigment, which in turn depends on the functional groups making up its structure.

A general separation of the acetone extract of pigments can be done on glass plates of 20×20 cm covered in the laboratory with silica gel 60 GF₂₅₄ to a thickness of 1 mm. These are air-dried and activated for 1 h in a stove at 120°C. If preferred, the plates can be acquired commercially from a wide variety of sources. The usual developer used is the mixture petroleum ether (65 to 95°C)–acetone–diethylamine (10:4:1) (System I) (Mínguez-Mosquera and Garrido-Fernández, 1989). The development chamber must be saturated, and the solvent must rise to three quarters the height of the plate, which usually takes between 15 and 30 min.

This system does not allow separation of the individual dephytylated chlorophyll derivatives, which, due to their high polarity, are all retained at the base of the chromatogram ($R_f = 0$). This drawback is circumvented either by modifying the polarity of developer I with a substitution of diethylamine by pyridine at a higher proportion (10:4:2.5) (System II) (Mínguez-Mosquera et al., 1989b), or by using reversed-phase chromatography. In this case, kieselgühr plates impregnated with maize oil (14%) or commercial plates of silica gel C₁₈ are used, with the eluent mixture methanol–acetone–water (20:4:6) (System III) (Jones et al., 1972; Mínguez-Mosquera et al., 1993). The recovery of these compounds from the adsorbent is very difficult because the usual solvents do not completely solubilize them. Their elution is achieved with the mixture acetone–pyridine (1:1) (Mínguez-Mosquera et al., 1989b). If some pigments coincide in R_f value under these conditions, specific eluent systems are used for their individual separation (Table 4.2).

HPLC can also be used in the isolation of the different standard chlorophyll derivatives in their pure form, employing a semipreparative column of 25 cm in length and 1 cm internal diameter (I.D.) (Mínguez-Mosquera et al., 1996). Each fraction collected in semipreparative HPLC is transferred to ethyl ether, and aqueous NaCl solution

(10%) is added. The solvent is removed in a rotavapor, and each pure pigment fraction is subjected to different identification tests. This chromatographic development in reversed phase separates the pigments in direct relation to their polarity. The retention of a compound in HPLC is expressed by the capacity factor k , defined as

$$k = (t_r - t_m)/t_m$$

where t_r is the retention time of each component and t_m is the time taken to elute the nonretained solvent. However, the most practical way to express the retention of a compound in HPLC is referring to that of a standard substance (Yost et al., 1980):

$$\text{relative retention } (r_{i,st}) = k_i/k_{st}$$

where the subscripts i and st refer to the respective values for the peak of the component under study and that of the substance chosen as the standard.

4.3.4 IDENTIFICATION

Having proven the purity of each pigment, the next stage is to study its physico-chemical characteristics. The typical coloration shown by these compounds in TLC under white and UV_{254 nm} light and the corresponding R_f values in TLC, in both normal and reversed phase, are the first guiding data in their characterization. Subsequently, absorption spectra are obtained in different solvents, whose dielectric properties affect the spectrum. The shape, location of the absorption maxima, and the ratio between the Soret band and the maximum in the red region contribute very valuable information about the molecular structure of the compound. Finally, cochromatography with the corresponding standards, acquired commercially or prepared in the laboratory as detailed previously, adds to the identification. Tables 4.2 and 4.3 summarize these characteristics for the different pigments.

Table 4.4 shows the chromatographic ($r_{i,st}$) and spectroscopic (electron absorption maxima in the eluent) characteristics obtained from the separation of chlorophylls and derivatives by HPLC and UV-Vis detection. Pigment identification is completed with assignation of IR spectrum bands and mass spectrum.

4.3.5 OBTAINING OF STANDARDS

Chlorophylls *a* and *b* — A widely used source of chlorophylls *a* and *b* is spinach leaves, due to the abundant presence of these compounds and their easy isolation. This is carried out by extraction of the pigments with acetone, followed by the separation and purification from other pigments by TLC on silica gel with the eluent mixture petroleum ether (65° to 95°C) — acetone — diethylamine (10:4:1) (Holden, 1976; Mínguez-Mosquera and Garrido-Fernández, 1985). Standard chlorophyll *a* and chlorophyll *b* can be obtained commercially, for instance, from Sigma Chemical Co. (St. Louis, MO).

TABLE 4.2
Chromatographic Characteristics in TLC of Chlorophylls and Derivatives

Pigment ^b	R _f Values ^a				
	I	II	III	IV	V
Frequent					
Chlorophyll <i>a</i>	0.51	—	0.00	—	—
Chlorophyll <i>b</i>	0.44	—	0.00	—	—
Chlorophyll <i>c</i>	0.00	0.00	—	—	—
Pheophytin <i>a</i>	0.57	0.91	0.00	0.48	—
Pheophytin <i>b</i>	0.53	0.87	0.00	0.33	—
Chlorophyllide <i>a</i>	0.00	0.36	0.75	—	—
Chlorophyllide <i>b</i>	0.00	0.24	0.96	—	—
Pheophorbide <i>a</i>	0.00	0.45	0.30	—	0.69
Pheophorbide <i>b</i>	0.00	0.34	0.48	—	0.75
Pyropheophytin <i>a</i>	0.63	—	—	0.67	—
Pyropheophytin <i>b</i>	0.51	—	—	0.45	—
Pyropheophorbide <i>a</i>	—	—	—	—	0.58
Pyropheophorbide <i>b</i>	—	—	—	—	0.62
Not frequent					
Chlorin <i>e</i> ₆	0.00	—	—	—	0.84
Rhodin <i>g</i> ₇	0.00	—	—	—	0.88
15 ¹ -OH-lactone-pheophytin <i>a</i>	0.00	0.63	—	—	—
15 ¹ -OH-lactone-pheophytin <i>b</i>	0.00	0.59	—	—	—
15-Glyoxylic acid pheophytin <i>a</i>	0.00	0.56	—	—	—
15-Glyoxylic acid pheophytin <i>b</i>	0.00	0.41	—	—	—
Zn-pheophytin <i>a</i>	0.56	—	—	0.49	—
Zn-pheophytin <i>b</i>	0.55	—	—	0.34	—
Zn-pyropheophytin <i>a</i>	0.61	—	—	0.68	—
Zn-pyropheophytin <i>b</i>	0.51	—	—	0.46	—
Cu-pheophytin <i>a</i>	0.57	0.91	—	0.43	—
Cu-pheophytin <i>b</i>	0.55	0.87	—	0.30	—
Cu-pyropheophytin <i>a</i>	0.64	—	—	0.65	—
Cu-pyropheophytin <i>b</i>	0.52	—	—	0.40	—
Cu-15-glyoxylic acid pheophytin <i>a</i>	0.00	0.48	—	—	—
Cu-15-glyoxylic acid pheophytin <i>b</i>	0.00	0.46	—	—	—

^a Eluents: I. Petroleum ether (65°–95°C)–acetone–diethylamine (10:4:1); II. Petroleum ether (65°–95°C)–acetone–pyridine (10:4:2.5); III. Methanol–acetone–water (20:4:6); IV. Hexane–acetone–diethylamine (7:4:0.5); V. Methanol–acetone–water (20:4:3). Adsorbents: Systems I, II, and IV: silica gel 60 GF₂₅₄; System III: kieselgur (with fluorescent indicator UV₂₅₄) impregnated with 14% (v/v) of maize oil in light petroleum (40°–60°C); System V: silica gel C₁₈ (HPTLC plates).

^b Frequent: pigments frequently found in foods of plant origin. Not frequent: pigments occasionally found in foods of plant origin.

TABLE 4.3
Color on Plate in TLC and Spectroscopic Characteristics in Different Solvents of Chlorophylls and Derivatives

Pigment ^b	Color on Plate		Spectral Data: λ_{max} ^a					
	Daylight	UV ₃₆₆ Radiation ^c	Acetone		Diethyl Ether		Acetone–Pyridine (1:1)	
Frequent								
Chlorophyll <i>a</i>	Blue-green	Red F	(384) 408	428 536 580 616 662	(382) 410	430 533 578 615 662		
Chlorophyll <i>b</i>	Yellow-green	Red F	(432) 454 595 646		(430) 456 549 595 644			
Chlorophyll <i>c</i>	Yellow-green	Red F	444 580 630		438 579 630			
Pheophytin <i>a</i>	Grey	Red F	408 466 504 534 554 608 666		408 471 505 534 609 668			
Pheophytin <i>b</i>	Brown	Red F	(414) 430 523 602 656		(412) 434 525 555 599 655			
Chlorophyllide <i>a</i>	Blue-green	Red F	(384) 408	428 536 580 616 662			(390) 420 442 618 640 670	
Chlorophyllide <i>b</i>	Yellow-green	Red F	(432) 454 595 646				(446) 470 556 502 654	
Pheophorbide <i>a</i>	Grey	Red F	408 466 504 534 554 608 666				412 470 506 536 610 668	
Pheophorbide <i>b</i>	Brown	Red F	(414) 430 523 602 656				(438) 526 556 600 656	
Pyropheophytin <i>a</i>	Grey	Red F	408 466 504 534 554 608 666		408 471 505 534 609 668			
Pyropheophytin <i>b</i>	Brown	Red F	(414) 430 523 602 656		(412) 434 525 555 599 655			
Pyropheophorbide <i>a</i>	Grey	Red F	408 466 504 534 554 608 666				412 470 506 536 610 668	
Pyropheophorbide <i>b</i>	Brown	Red F	(414) 430 523 602 656				(438) 526 556 600 656	

Not Frequent

Chlorin e_6	Grey	Red F	400 500 (532) 602 660	400 500 (530) 560 610 666
Rhodin g_7	Brown	Red F	(408) 426 (530) (570) (596) 648	(408) 427 (524) (560) (597) 652
15'-OH-lactone- pheophytin a	Grey	Red F	398 498 530 612 668	
15'-OH-lactone- pheophytin b	Brown	Red F	(414) 424 518 (556) (596) 648	
15-Glyoxylic acid pheophytin a	Grey	Red F	400 500 (532) 602 660	
15-Glyoxylic acid pheophytin b	Brown	Red F	(408) 426 (530) (570) (596) 648	
Zn-pheophytin a	Green	Red F		(376) (406) 425 522 564 606 656
Zn-pheophytin b	Green	Red F		445 637
Zn-pyropheophytin a	Green	Red F		(376) (406) 425 522 564 606 656
Zn-pyropheophytin b	Green	Red F		445 637
Cu-pheophytin a	Green	No F		398 422 504 548 606 650
Cu-pheophytin b	Green	No F		438 520 584 628
Cu-pyropheophytin a	Green	No F		398 422 504 548 606 650
Cu-pyropheophytin b	Green	No F		438 520 584 628
Cu-15-glyoxylic acid pheophytin a	Blue	No F	(398) 406 502 (598) 630	
Cu-15-glyoxylic acid pheophytin b	Yellow-green	No F	428 566 614	

^a The values in parentheses indicate inflection points in the absorption spectrum.

^b Same as in Table 4.2.

^c F, fluorescence.

TABLE 4.4
Chromatographic and Spectroscopic Characteristics in HPLC of Chlorophyll
and Derivatives

Pigment ^b	Spectral Data in the HPLC Eluent: λ_{max}^a						
	Soret	I	II	III	IV	V	R
Frequent							
Chlorophyllide <i>b</i>	0.20					600	3.3
Chlorophyllide <i>a</i>	1.11	(384)	(412)		(580)	616	1.3
Chlorophyll <i>c</i>	2.02					582	7.0
Pheophorbide <i>b</i>	2.10		(412)	524	(558)	598	4.3
Pheophorbide <i>a</i>	3.09	(400)	(478)	506	534	608	1.8
Pyro-pheophorbide <i>b</i>	4.04		(412)	524	(558)	598	4.3
Pyro-pheophorbide <i>a</i>	4.50	(400)	(478)	506	534	608	1.8
Chlorophyll <i>b</i>	7.96					600	3.3
Chlorophyll <i>b'</i>	8.24					600	3.3
Chlorophyll <i>a</i>	9.18	(384)	(412)		(580)	616	1.3
Chlorophyll <i>a'</i>	9.47	(384)	(412)		(580)	616	1.3
Pheophytin <i>b</i>	11.21		(412)	524	(558)	598	4.3
Pheophytin <i>b'</i>	11.91		(412)	524	(558)	598	4.3
Pheophytin <i>a</i>	13.22	(400)	(478)	506	534	608	1.8
Pheophytin <i>a'</i>	13.75	(400)	(478)	506	534	608	1.8
Pyro-pheophytin <i>b</i>	13.90		(412)	524	(558)	598	4.3
Pyro-pheophytin <i>a</i>	16.05	(400)	(478)	506	534	608	1.8

Not frequent

Rhodin g_7	0.17	0.02	426	(408)	(530)	(570)	(596)	648	6.5
Chlorin e_6	0.72	0.08	400		500	(532)	602	660	3.8
15-Glyoxylic acid pheophytin b	6.47	0.70	426	(408)	(530)	(570)	(596)	651	6.5
15-Glyoxylic acid pheophytin a	7.21	0.79	400		500	(532)	607	662	2.9
15'-OH-lactone-pheophytin b	8.46	0.92	427		(416)	(560)	(600)	651	4.5
Zn-pheophytin b	8.89	0.97	456				590	640	3.1
Zn-pyro-pheophytin b	9.84	1.07	456				590	640	3.1
Zn-pheophytin a	9.93	1.08	428	(376)	(410)	(560)	610	656	1.2
15'-OH-lactone-pheophytin a	10.33	1.13	400		499	531	614	670	2.6
Zn-pyro-pheophytin a	10.58	1.15	428	(376)	(410)	(560)	610	656	1.2
Cu-pheophytin b	11.10	1.21	428			(564)	612	5.6	
Cu-pheophytin a	14.17	1.54	424	(384)	400	506	606	654	1.0
Cu-pyro-pheophytin b	14.41	1.57	428			(564)	612	5.6	
Cu-pyro-pheophytin a	17.75	1.93	424	(384)	400	506	606	654	1.0

^a R , quotient of absorbance at Soret band divided by absorbance at wavelength VI. The values in parentheses indicate inflection points in the absorption spectrum.

^b Same as in Table 4.2.

^c Retention factor, $k_c = (t_R - t_M)/t_M$, where t_R is the retention time of the pigment peak and t_M is the retention time of an unretained component.

^d Relative retention, $r_{\text{rel}} = k_i/k_{\text{st}}$; st = chlorophyll a .

Chlorophyll *c* — This is obtained from an acetone extract of pigments from alga of the genus *Phaeodactylum*. They are separated and purified by TLC under the same conditions as chlorophylls *a* and *b*. The strong adsorption of this pigment on the support requires elution from the silica with acetone — pyridine (1:1) (Mínguez-Mosquera, 1991c).

Pheophytins *a* and *b* — These are prepared from the respective pure solutions of chlorophylls in ethyl ether by acidification with 2 to 3 drops of 13% HCl (v/v). The mixture is shaken for 5 min, and the acid is removed by washing with 2% Na₂SO₄ solution. Finally, the ether phase is dried by filtration through a bed of anhydrous Na₂SO₄ (Holden, 1976; Sievers and Hynninen, 1977).

Chlorophyllides *a* and *b* — Incubation of pure solutions of the corresponding chlorophylls with active enzymatic extract (chlorophyllase) de-esterifies the chlorophylls to form chlorophyllides. Fresh leaves of *Ailanthus altissima*, whose tissue is considered one of the richest sources of chlorophyllase (McFeeters et al., 1971), are used as the biological material. The protein precipitate is obtained starting with 5 g of fresh, chopped leaves. Acetone (100 ml at -20°C) is added, and the mixture is triturated using Ultraturrax in a crushed-ice bath to keep the temperature down and to minimize solubilization of the enzyme. The homogenization is carried out for 1 min at the lowest possible rate to prevent the formation of foam, which is a symptom of enzyme denaturalization. The residue is collected by filtration *in vacuo* and treated with acetone until the extracts are colorless. Normally, four extractions are sufficient. The protein precipitate obtained is left to dry at room temperature and stored at -20°C until use. The conversion of chlorophylls to chlorophyllides is carried out under the optimum conditions of enzyme activation. The reaction is performed in glass flasks with PVL screw caps and a Teflon washer. Pure solutions of chlorophyll *a* or chlorophyll *b* in acetone are incubated for 24 h at 30°C with 0.5 g of acetone powder and chlorophyllase buffer such that the final concentration of acetone is 50%. The chlorophyllase buffer is tris-20 mM HCl at pH 8, which also contains 0.5 M NaCl and 10 mM MgCl₂ (Terpstra and Lambers, 1983). At the end of incubation, the pigments are transferred to ethyl ether and completion of the reaction is verified by TLC on silica gel (Mínguez-Mosquera et al., 1989b).

Pheophorbides *a* and *b* — These can be prepared similarly to pheophytins by acidification of the corresponding ether solution of chlorophyllide or similarly to chlorophyllides by enzymatic de-esterification of the respective pheophytins.

C₁₀ epimers — These are formed by dissolving the corresponding pigment in chloroform and keeping the solution in the refrigerator for 2 h (Watanabe et al., 1984).

Pyropheophytins and pyropheophorbides *a* and *b* — These are prepared from the corresponding pheophytins and pheophorbides by heat treatment of these compounds in pyridine at 100°C for 24 h with reflux (Schwartz et al., 1981).

Cu pheophytins and pheophorbides *a* and *b* — The Cu-chlorophyll complexes are obtained by adding 20 ml of 1 M CuCl₂ to a solution of the corresponding pheophytin in acetone (80 ml). Ascorbic acid crystals are added to the reaction mixture to prevent oxidative changes, and the chelation is kept under N₂ atmosphere for 2 h, as described by Jones et al. (1968).

Zn pheophytins and pheophorbides *a* and *b* — These are prepared similarly to the Cu complexes, except that crystals of ZnCl_2 (5 g) are added directly to the respective solution of pheophytin in acetone (80 ml), and the mixture is stirred magnetically for 2 h (Jones et al., 1968).

Methyl esters of chlorin *e*₆ and rhodin *g*₇ — These are formed by saponification of pheophorbide *a* and pheophorbide *b*, respectively, with a solution of 0.5% KOH in methanol (Hynninen, 1973).

Free chlorin *e*₆ and rhodin *g*₇ — These are obtained from their respective methyl esters by saponification with methanolic solution of KOH (30%) at room temperature and under a nitrogen atmosphere (Hynninen, 1973).

Purpurins 18a and b — These are obtained by oxidation of pheophorbide *a* and *b*, respectively, by alkaline treatment with 30% KOH solution in methanol in the presence of atmospheric oxygen (Hynninen, 1973).

Allomerized chlorophylls — These are prepared by dissolving 1 to 2 μmol of chlorophyll *a* or *b* in 5 ml of methanol and keeping the solution at room temperature and in darkness with magnetic stirring for 3 days. The progress of the reaction is followed by HPLC (Kuronen et al., 1993).

4.3.6 QUANTIFICATION

4.3.6.1 Quantitative Determination of Pigments by UV-Vis Spectrophotometry

The simplest case is an overall evaluation of the chlorophyll pigments contained in a sample. For this, the starting point is a total extract of pigments, which is analyzed spectrophotometrically at the wavelength of maximum absorption in the red region. Such quantification is feasible because in this region of the spectrum, specifically at 662 to 666 nm, there is no absorption of the carotenoid pigments that normally accompany the chlorophyll pigments in extracts. This method is the one most used in control laboratories. Its foremost advantage is that measurement is almost immediate and requires minimum sample treatment and no analytical operation (with the exception of absorbance measurement). Its basic disadvantage is that quantification assumes a theoretical major pigment, which may not always be the case.

The relationship between the molar concentration of a chlorophyll solution and the optic density or absorbance at a particular wavelength (λ) fits a linear response defined by the Beer–Lambert law:

$$E = \epsilon \times C \times L$$

where

E = optic density, absorbance, or extinction of the sample at λ

C = molar concentration of pigment

L = optic pathway through the solution (cm)

ϵ = molar coefficient of extinction at λ

The intensity of an absorption band can also be expressed as *specific coefficient of extinction*, $\epsilon^{1\%}$, defined as the absorbance of 1 g of pigment in 100 ml of a specific solvent at a specific λ .

The relationship between the two coefficients of extinction is the following:

$$\epsilon (\text{M}^{-1} \times \text{cm}^{-1}) = 0.1 \times, \epsilon^{1\%} \times \text{MW}$$

where MW is the molecular weight. The values of both coefficients for each pigment are available in the References (Scheer, 1988).

The evaluation method according to this technique is as follows: the pigment extract is taken to a known volume with a solvent appropriate for spectrophotometric measurement (ethanol, acetone, etc.). The absorbance at the wavelength of measurement must be between 0.2 and 0.8, and quantification is performed adapting the formula of the Beer–Lambert law:

$$C = \frac{E \times V_f}{\epsilon^{1\%} \times W_s} \times 10^4$$

where

C = concentration (mg/kg)

V_f = final volume of pigment extract (ml)

W_s = sample weight (g)

$\epsilon^{1\%}$ = specific extinction of a 1% solution (1 g in 100 ml) measured with an optical pathway of 1 cm

E = extinction at λ of measurement

Normally, in a complex solution of pigments, $\epsilon^{1\%}$ is arbitrarily taken as that of the major pigment. The results are expressed as milligrams per kilogram of sample.

4.3.6.2 Quantitative Determination of Pigments by TLC and UV-Vis Spectrophotometry

At the end of the TLC development of an extract containing pigments previously identified, each component identified is scraped from the plate individually and eluted with acetone to a determinate volume whose absorbance lies between 0.2 and 0.8. The value of extinction E , at the wavelength of maximum absorption, is substituted in the Beer–Lambert equation. After the appropriate operations to express the result in milligrams per kilogram of sample, the resulting equation is

$$C = \frac{E \times V_i \times V_f}{\epsilon^{1\%} \times W_s \times V_{cr}}$$

where

C = concentration (mg/kg)

V_i = initial volume of pigment extract (ml)

V_f = final volume of elution (ml)

W_s = weight of sample (g)

V_{cr} = volume chromatographed (ml)

The coefficients of extinction in acetone for each pigment are calculated from those given in the References for chlorophylls and pheophytins in ethyl ether (Smith and Benítez, 1955) by using a pigment solution of known concentration. It is assumed that the coefficients of extinction of compounds with different chemical structure, but identical electron absorption spectrum (for example, chlorophyll/chlorophyllide), do not differ significantly (Jones et al., 1962). Thus, the specific coefficient of extinction ($\epsilon^{1\%}$) can be calculated from a known molar coefficient (ϵ_i) of another compound (i) with an identical spectrum:

$$\epsilon^{1\%} = \epsilon_i / 0.1 \times \text{MW}$$

Table 4.5 lists those coefficients.

4.3.6.3 Quantitative Determination of Pigments by HPLC and UV-Vis Spectrophotometry

4.3.6.3.1 Separation of Pigments

The proposed chromatographic system considers jointly both the pigments normally found in green tissues and the possible degradation products resulting from the various technological treatments. Pigment separation is achieved by using a reversed-phase C_{18} column (Spherisorb ODS-2) of 25 cm in length, 4.6 mm I.D., and 5 μm particle size, protected with a precolumn of 3 cm $\times$ 4 mm packed with the same material. The eluents used are (A) water–ion pair reagent–methanol (1:1:8) and (B) acetone–methanol (1:1). The ion pair reagent consists of a solution of tetrabutylammonium acetate (0.05 *M*) and ammonium acetate (1 *M*) in water. The addition of these salts to the eluent improves separation of the de-esterified chlorophyll derivatives (Mantoura and Llewellyn, 1983). After use, the column is stored in methanol–water (1:1) to prevent deterioration of the silica particles by the ionic reagent.

Before separating the acetone extract of pigments by HPLC, the sample is filtered through a nylon membrane of 0.45 μm pore diameter or centrifuged at 12000 *g*. Then it is injected (20 μl loop), and eluted at a flow rate of 2 ml/min (initial pressure 3800 psi) with the following gradient scheme:

Time (min)	% A	% B	Curve
Initial	75	25	
8	25	75	Linear
10	25	75	Isocratic
18	10	90	Convex
23	0	100	Concave
30	75	25	Concave

Depending on the pump model, the convex or concave curve profile may be modified, and therefore, those steps would need optimization. Detection is carried out at 430 nm using a programmable diode array detector (Mínguez-Mosquera et al., 1991b). Figure 4.12 shows the typical chromatogram for the separation of pigments

TABLE 4.5
Specific Absorption Coefficients
of Chlorophylls and Derivatives

Pigment ^a	$\lambda_{\max}$	$\epsilon_{\lambda_{\max}}^{1\%}$	$\epsilon_{430\text{ nm}}^{1\% \text{ b}}$
Frequent			
Acetone			
Chlorophyll <i>a</i>	428	840	840
Chlorophyll <i>b</i>	454	1450	356
Chlorophyll <i>c</i>	446	1400	—
Pheophytin <i>a</i>	406	1290	268
Pheophytin <i>b</i>	434	2060	1545
Pyropheophytin <i>a</i> ^c	406	1382	287
Pyropheophytin <i>b</i> ^c	434	2205	1653
Acetone — Pyridine			
Chlorophyllide <i>a</i>	438	1181	1220
Chlorophyllide <i>b</i>	468	2122	514
Pheophorbide <i>a</i>	410	1818	394
Pheophorbide <i>b</i>	436	3027	2254
Pyropheophorbide <i>a</i> ^c	410	2015	437
Pyropheophorbide <i>b</i> ^c	436	3347	2492
Not Frequent			
Chlorin <i>e</i> ₆ ^d	—	—	192
Rhodin <i>g</i> ₇ ^d	—	—	2775
15 ¹ -OH-lactone-pheophytin <i>a</i> ^d	—	—	127
15 ¹ -OH-lactone-pheophytin <i>b</i> ^d	—	—	1848
15-Glyoxylic acid pheophytin <i>a</i> ^d	—	—	129
15-Glyoxylic acid pheophytin <i>b</i> ^d	—	—	1877
Ethyl Ether^e			
Zn-pheophytin <i>a</i>	423	1335	803
Zn-pheophytin <i>b</i>	446	1869	568
Zn-pyropheophytin <i>a</i> ^c	423	1424	856
Zn-pyropheophytin <i>b</i> ^c	446	1991	606
Cu-pheophytin <i>a</i>	421	991	646
Cu-pheophytin <i>b</i>	438	1333	1333
Cu-pyropheophytin <i>a</i> ^c	421	1057	689
Cu-pyropheophytin <i>b</i> ^c	438	1420	1420
Cu-15-glyoxylic acid pheophytin <i>a</i> ^d	—	—	60
Cu-15-glyoxylic acid pheophytin <i>b</i> ^d	—	—	1308

^a Same as in Table 4.2.

^b Values of $\epsilon_{430\text{ nm}}^{1\%}$ are calculated from the absorption spectrum obtained with the photodiode array detector and from the values of $\epsilon_{\lambda_{\max}}^{1\%}$.

^c Values of $\epsilon^{1\%}$ are calculated from the molar absorbance coefficients of the respective pheophytins.

^d Values based on molar absorbance coefficients of the respective pheophytins.

^e Values according to Jones et al., 1968.

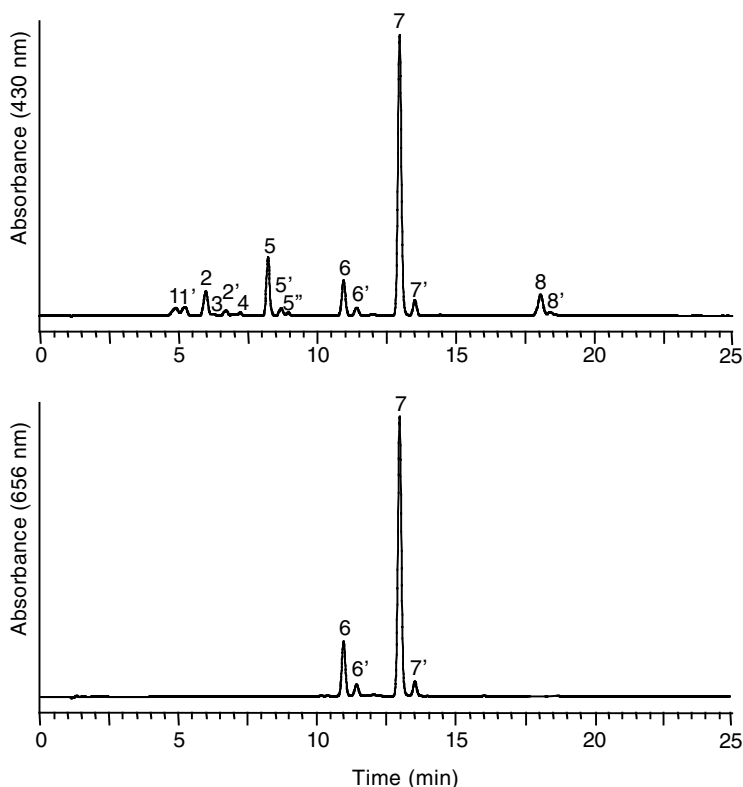


FIGURE 4.12 HPLC separation of pigments present in a green tissue. Peaks: 1, neoxanthin; 1', neoxanthin isomer; 2, violaxanthin; 2', violaxanthin isomer; 3, luteoxanthin; 4, anteraxanthin; 5, lutein; 5' and 5'', lutein isomers; 6, chlorophyll *b*; 6', chlorophyll *b* C-13² epimer; 7, chlorophyll *a*; 7', chlorophyll *a* C-13² epimer; 8, β -carotene; 8', cis β -carotene isomer.

from a green tissue, and Figure 4.13 shows that for a mixture of standards of the possible pigments present in an extract of algal origin. The following figures show the chlorophyllic and carotenoid pigments separation obtained in different processed vegetable products: frozen peas (Figure 4.14), canned peas (Figure 4.15), cucumbers in brine (Figure 4.16), virgin olive oil (Figure 4.17). In addition, Figure 4.18 shows a mixture of standards of oxidized pheophytins (Mínguez-Mosquera et al., 1996). If the pigment mixture contains carotenoids, their possible interference can be resolved by detecting the chlorophyll pigments at 656 nm, a wavelength at which the other pigments do not absorb.

The chromatogram obtained on separating pheophytins *a* and *b* from their respective Cu and Zn complexes (Figure 4.19) (Mínguez-Mosquera et al., 1995) enables detection of a possible alteration or adulteration of food color. As can be deduced from the retention times of these complexes, their separation from the rest of the pigments is perfectly practicable.

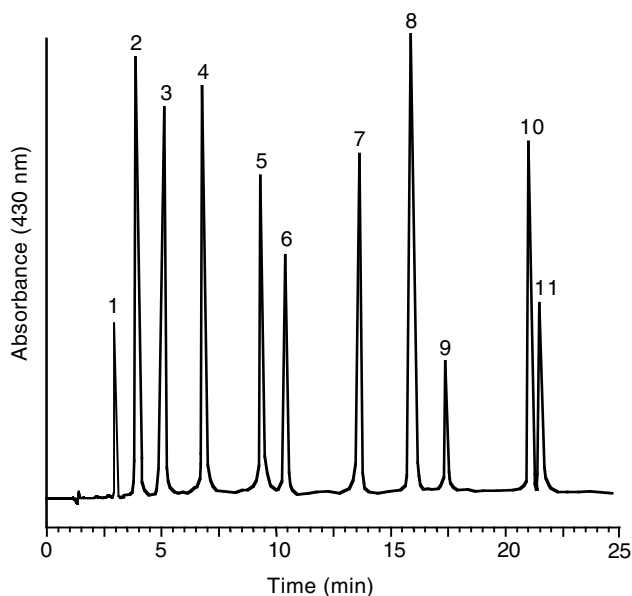


FIGURE 4.13 HPLC separation of a mixture of standards of possible pigments present in an extract of algal origin. Peaks: 1, chlorophyllide *a*; 2, chlorophyll *c*; 3, pheophorbide *a*; 4, fucoxanthin; 5, diadinoxanthin; 6, lutein; 7, chlorophyll *b*; 8, chlorophyll *a*; 9, asteroidenone; 10, β -carotene; 11, pheophytin *a*.

4.3.6.3.2 Quantification of Pigments

The best method for performing chromatographic quantification is the use of an internal standard, which must be a pigment absent from the material under study and separable from the other pigments. The useful standard pigments have been described in Chapter 3. Calibration and response factors calculations are carried out in the same way.

If an assay with an internal standard such as β -apo-8'-carotenal or canthaxanthin does not achieve a satisfactorily sharp separation between it and the chlorophyll or carotenoid pigments, other strategies will have to be adopted for the calibration. An external standard can be used, involving the preparation of a standard mixture in which the concentration of each component is known. This mixture is injected periodically to check possible instrumental variations. The calibration curve of each component is obtained by injecting different amounts of each pure pigment and making a linear fit from the plot of each peak area against the amount injected. At the same time, calibration curves in function of peak height are obtained in order to calculate the approximate limit of detection for each pigment, taking as the lower limit of detection the peak height equal to twice the noise signal.

The slow and laborious obtaining of standards for chlorophyll derivatives in the laboratory, together with the unreliable conservation of the standard mixtures due to the sensitivity of these pigments to light, temperature, etc., limit the use of this

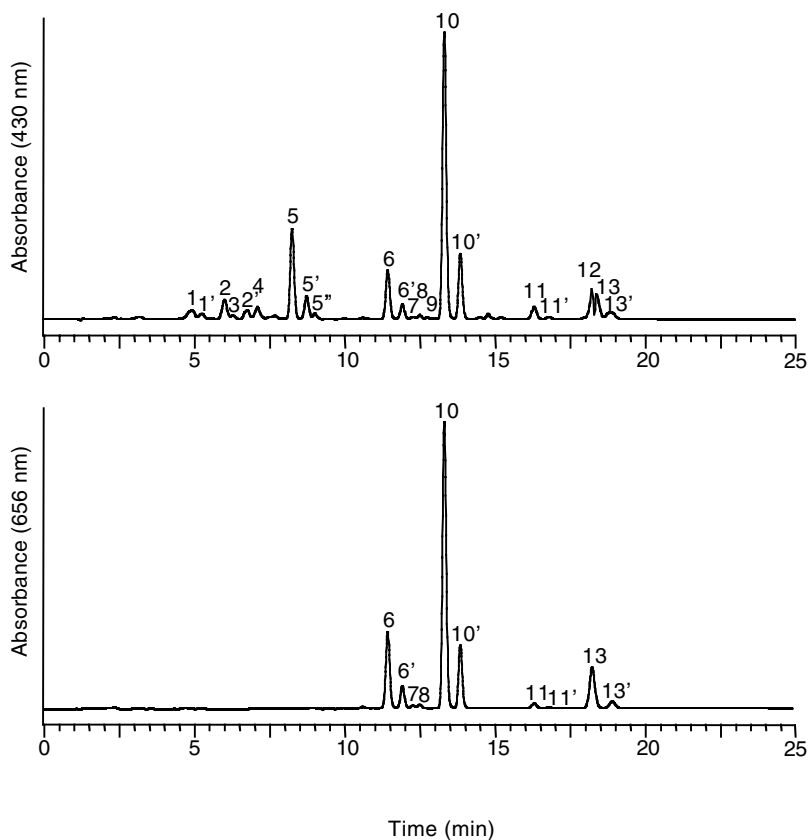


FIGURE 4.14 HPLC separation of pigments present in frozen peas. Peaks: 1, Neoxanthin, 1', neoxanthin isomer; 2, violaxanthin, 2', violaxanthin isomer; 3, luteoxanthin; 4, anteraxanthin; 5, lutein, 5' and 5'', lutein isomers; 6, chlorophyll *b*, 6', chlorophyll *b* C-13² epimer; 7, OH-lactone-chlorophyll *a*; 8, OH-chlorophyll *a*; 9, β -cryptoxanthin; 10, chlorophyll *a*, 10', chlorophyll *a* C-13² epimer; 11, pheophytin *b*; 11', pheophytin *b*, C-13² epimer; 12, β -carotene; 13, pheophytin *a*, 13' pheophytin *a* C-13² epimer.

method of quantification. The chlorophylls are degraded very readily to pheophytins, totally changing their spectrophotometric characteristics. Consequently, the certainty that the standard mixture is at its initial concentration is quickly lost. This means the continual preparation of pure pigments, excessively lengthening the analysis time. The best way to avoid this complication is to use another method that estimates the concentrations of the components from an extension of the Beer–Lambert law, adapted to nonuniform systems (Torsi et al., 1992), and governed by the equation

$$N_o = A_i \times F/\epsilon_\lambda \times L \times 10^3$$

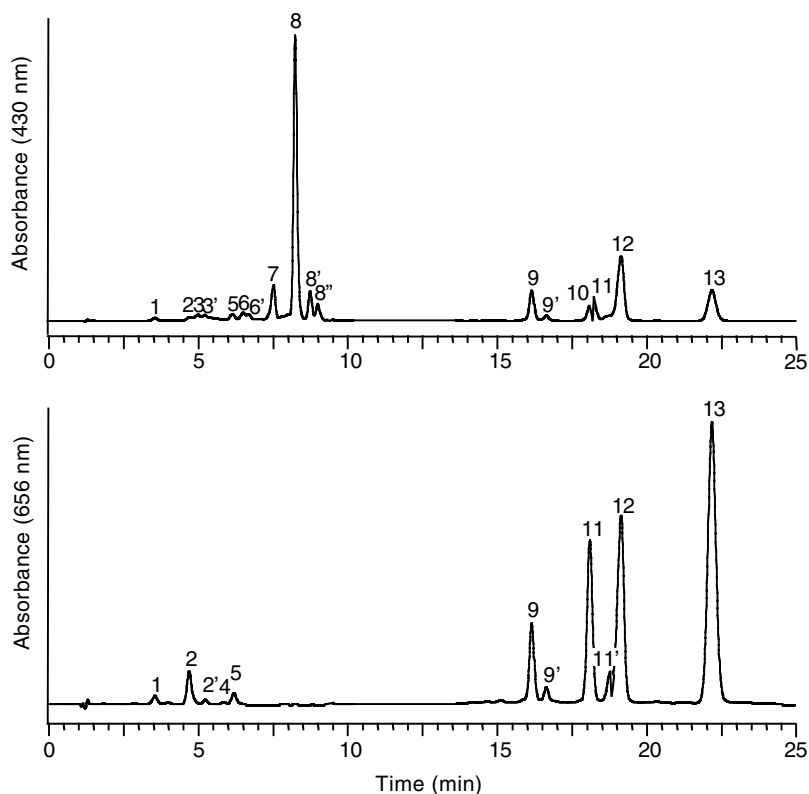


FIGURE 4.15 HPLC separation of pigments present in canned peas. Peaks: 1, pheophorbide *b*; 1', pheophorbide *b* C-13² epimer; 2, pheophorbide *a*; 2', pheophorbide *a* C-13² epimer; 3, neochrome; 3', neochrome isomer; 4, pirophorbide *b*; 5, pirophorbide *a*; 6, auroxanthin; 6', auroxanthin isomer; 7, mutatoxanthin; 8, lutein; 8' and 8'', lutein isomers; 9, pheophytin *b*; 9', pheophytin *b* C-13² epimer; 10, β -carotene; 11, pheophytin *a*; 11', pheophytin *a* C-13² epimer; 12, pirophophytin *b*; 13, pirophophytin *a*.

where

N_o = number of moles per square centimeter

A_i = peak area = absorbance $\times$ time (min)

F = flow rate (ml/min)

ϵ_λ = molar extinction at the λ of detection ($\text{cm}^2 \times \text{mol}^{-1}$)

L = length of cell (cm), specific to each detector

Making the appropriate conversions of units to express the result in milligrams per kilogram, we have

$$C = (A \times F \times V_f \times 10) / (\epsilon_\lambda^{1\%} \times V_i \times W_s \times L) \quad (4.1)$$

where

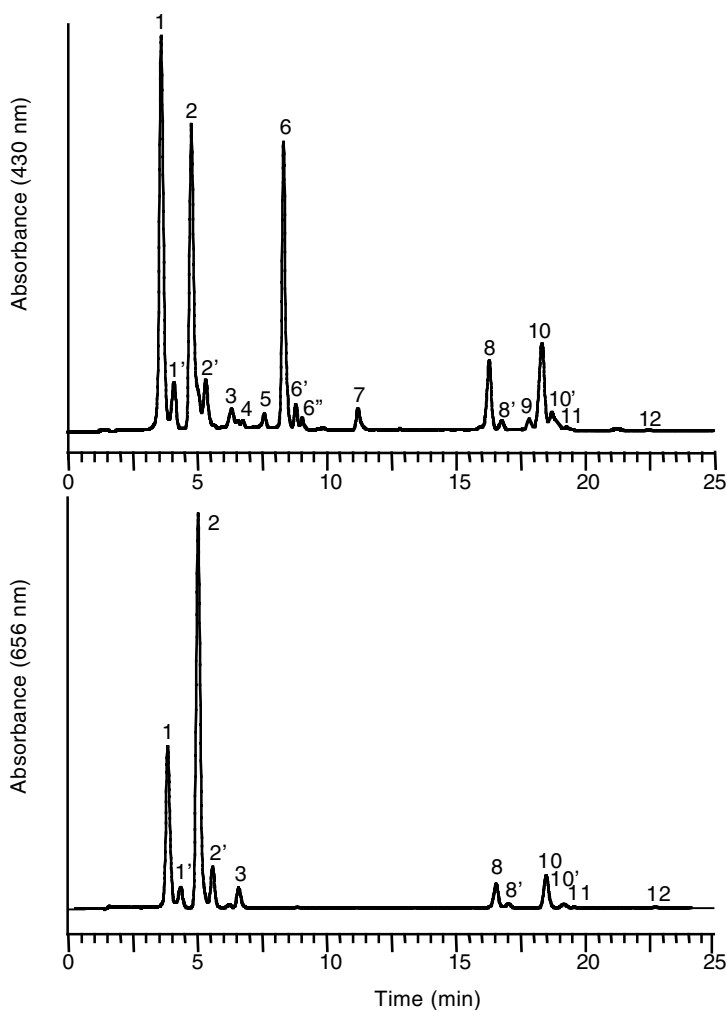


FIGURE 4.16 HPLC separation of pigments present in cucumbers in brine. Peaks: 1: pheophorbide *b*; 1', pheophorbide *b* C-13² epimer; 2, pheophorbide *a*; 2', pheophorbide *a* C-13² epimer; 3, pirophorbide *a*; 4, auroxanthin; 5, mutatoxanthin; 6, lutein; 6' and 6'', lutein isomers; 7, carotenoid; 8, pheophytin *b*; 8', pheophytin *b* C-13² epimer; 9, β -carotene; 10, pheophytin *a*; 10', pheophytin *a* C-13² epimer; 11, pirophophytin *b*; 12, pirophophytin *a*.

C = concentration (mg/kg)

A = peak area (absorbance $\times$ min)

F = flow rate (ml/min)

V_f = volume in which the extract is collected (ml)

$\epsilon_{\lambda}^{1\%}$ = specific extinction at the detection wavelength

V_i = volume of sample injected into the chromatograph (μ l)

W_s = weight of sample (g)

L = length of cell (cm)

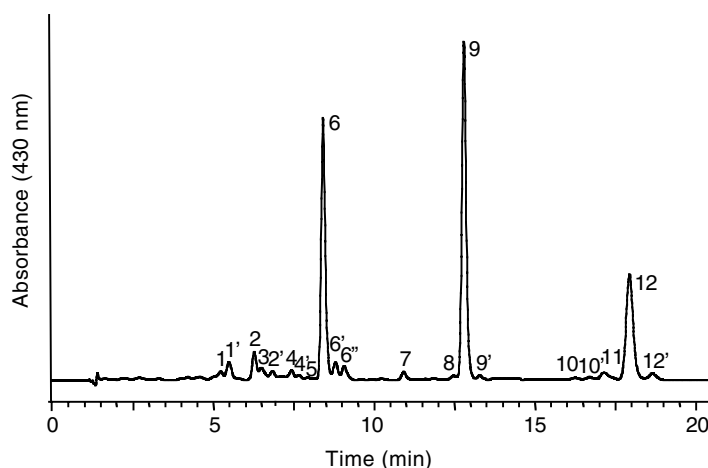


FIGURE 4.17 HPLC separation of pigments present in virgin olive oil. Peaks: 1, neoxanthin; 1', neoxanthin isomer; 2, violaxanthin; 2', violaxanthin isomer; 3, luteoxanthin; 4, anteraxanthin; 4', anteraxanthin isomer; 5, mutatoxanthin; 6, lutein; 6' and 6'' lutein isomers; 7, chlorophyll *b*; 8, β -criptoxanthin; 9, chlorophyll *a*; 9', chlorophyll *a*'; 10, pheophytin *b*; 10', pheophytin *b*'; 11, β -carotene; 12, pheophytin *a*; 12', pheophytin *a*'.

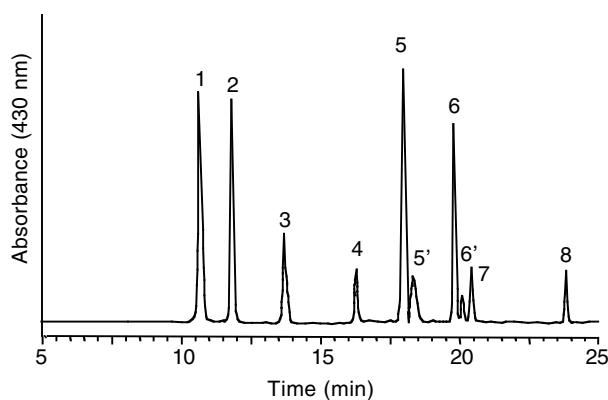


FIGURE 4.18 HPLC separation of a mixture of standards of pheophytins and their oxidation products. Peaks: 1, 15-glyoxilic acid pheophytin *b*; 2, 15-glyoxilic acid pheophytin *a*; 3, 15-OH-lactone-pheophytin *b*; 4, 15-OH-lactone-pheophytin *a*; 5, pheophytin *b*; 5', pheophytin *b* C-13² epimer; 6, pheophytin *a*; 6', pheophytin *a* 13² epimer; 7, purpurin 18 *b* phytol ester; 8, purpurin 18 *a* phytol ester.

The values of $\epsilon_{\lambda}^{1\%}$ are calculated for each pigment from the absorption spectrum obtained with the detector and from the values of $\epsilon_{\lambda, \max}^{1\%}$ given in the References (Mínguez-Mosquera et al., 1991b; Wright and Shearer, 1984). For a given concentration,

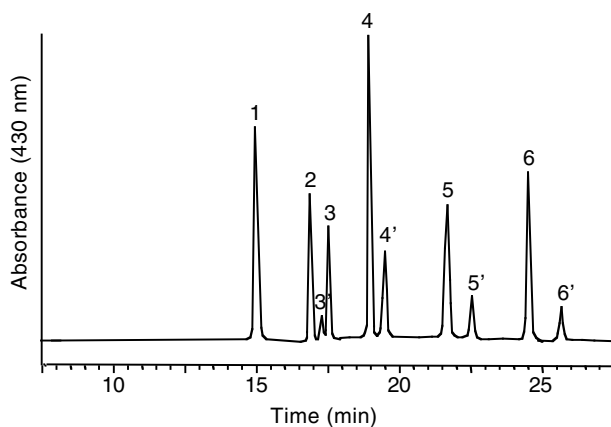


FIGURE 4.19 HPLC separation of pheophytins *a* and *b* from their respective Cu and Zn complexes. Peaks: 1, Zn-pheophytin *b*; 2, Zn-pheophytin *a*; 3, Cu-pheophytin *b*; 3', Cu-pheophytin *b* isomer; 4, pheophytin *b*; 4', pheophytin *b* C-13² epimer; 5, pheophytin *a*; 5', pheophytin *a* C-13² epimer; 6, Cu-pheophytin *a*; 6', Cu-pheophytin *a* isomer.

$$C = E_{\lambda} / \epsilon_{\lambda}^{1\%} = E_{\lambda_{\max}} / \epsilon_{\lambda_{\max}}^{1\%}$$

In practice, however, there may be some variations in instrumental sensitivity (deterioration of the lamp, changes in the index of refraction with elution gradient, etc.) that affect absorption of the light and thereby affect quantification. To control such changes, Equation 4.1 is multiplied by a correction factor (F_c) obtained from the calibration curves of chlorophyll *a*.

The proposed method enables a correct quantification of pigments, needing only as periodic control a solution of chlorophyll *a* as standard for the chlorophyll derivatives (Mínguez-Mosquera et al., 1991b).

REFERENCES

- Abaychi, J.K. and Riley, J.P., The determination of phytoplankton pigments by high-performance liquid chromatography, *Anal. Chim. Acta*, 107, 1, 1979.
- Almela, L., Fernández-López, J.A., and Roca, M.J., High-performance liquid chromatographic screening of chlorophyll derivatives produced during fruit storage, *J. Chromatogr.*, 870, 483, 2000.
- Anderson, A.F.H. and Calvin, M., An improved method for the separation and purification of chlorophyll *a*, *Nature*, 194, 285, 1962.
- AOAC Official Methods of Analysis, Chlorophyll in plants, *Method*, 3, 135, 59, 1984.
- Baber, M., Bordoni, R.S., Elliot G.J., Sedgwick, R.C., and Tyler, A.N., Fast atom bombardment mass spectrometry, *Anal. Chem.*, 54, 645, 1982.
- Bacon, M.F., Separation of chlorophylls *a* and *b* related compounds by thin-layer chromatography on cellulose, *J. Chromatogr.*, 17, 322, 1965.

- Bacon, M.F., Artifacts from chromatography of chlorophylls, *Biochim. J.*, 101, 34, 1966.
- Bacon, M.F. and Holden, M., Changes in chlorophylls resulting from various chemical and physical treatments of leaves and leaf extracts, *Phytochemistry*, 6, 193, 1967.
- Bauernfeind, J.C., Brubacher, G.B., Kläui, H.M., and Marusich, W.L., Use of carotenoids, in *Carotenoids*, Isler, O., Ed., Birkhäuser Verlag, Basel, Stuttgart, 1971, p. 743.
- Bazzaz, M.B. and Rebeiz, C.A., Chloroplast structure. V. Spectrofluorometric determination of chlorophyll(a) and b and pheophytin (or pheophorbide) a and b in unsegregated pigment mixtures, *Photochem. Photobiol.*, 30, 209, 1979.
- Berset, C. and Caniaux, P., Relationship between color evaluation and chlorophyll content in dried parsley leaves, *J. Food Sci.*, 48, 1854, 1983.
- Blanc, M., Chlorophyll pigments of tea; distribution and behaviour during black tea manufacture, *Food Sci. Tech. Abstr.*, 1h, 89, 1973.
- Boardman, N.K. and Thorne, S.W., Sensitive fluorescence method for the determination of chlorophyll a/chlorophyll b ratios, *Biochim. Biophys. Acta.*, 253, 222, 1971.
- Braumann, T. and Grimme, L.H., Single-step separation and identification of photosynthetic pigments by high-performance liquid chromatography, *J. Chromatogr.*, 170, 264, 1979.
- Brereton, R.G., Bazzaz, M.B., Santikarn, S., and Williams D.H., Positive and negative ion fast atom bombardment mass-spectrometric studies on chlorophylls: structure of 4-vinyl-4-desethyl chlorophyll b, *Tetrahedron Lett.*, 24, 5775, 1983.
- Bricker, D.L. and Russell, D.H., Study of target gas excitation accompanying collisional activation of large polyatomic ions, *J. Am. Chem. Soc.*, 108, 6174, 1986.
- Brown, L.M., Hargrave, B.T., and MacKinnon, M.D., Analysis of chlorophyll a in sediments by high-pressure liquid chromatography, *Can. J. Fish. Aquat.*, 38, 205, 1981.
- Brunisma, J., The quantitative analysis of chlorophylls a and b in plant extracts, *Photochem. Photobiol.*, 2, 241, 1963.
- Buckle, K.A. and Edwards, R.A., Chlorophyll degradation and lipid oxidation in frozen unblanched peas, *J. Sci. Food Agric.*, 21, 307, 1970.
- Buckle, K.A. and Rahman, F.M.M., Separation of chlorophyll and carotenoid pigments of *Capsicum* cultivars, *J. Chromatogr.*, 171, 385, 1979.
- Canjura F.L., Watkins, R.H., and Schwartz, S.J., Color improvement and metallo-chlorophyll complexes in continuous flow aseptically processed peas, *J. Food Sci.*, 64, 987, 1999.
- Chait, B.Y. and Field, F.H., ²⁵²Cf fission fragment ionization mass spectrometry of chlorophyll a, *J. Am. Chem. Soc.*, 106, 1931, 1984.
- Chan, A.S.K., Ellsworth, R.K., Perkins, H.J., and Snow, S.E., Purification of dihydroporphyrins for specific activity determination by thin-layer chromatography, *J. Chromatogr.*, 47, 395, 1970.
- Chernomorsky, S., Rancourt, R., Sahai, D., and Porets, R., Evaluation of commercial chlorophyllin copper complex preparations by liquid chromatography with photodiode array detection, *J. AOAC Int.*, 80, 433, 1997.
- Chernomorsky, S., Segelmen, A., and Poretz, R.D., Effect of dietary chlorophyll derivatives on mutagenesis and tumor cell growth, *Teratogenesis Carcinogen. Mutagen*, 19, 313, 1999.
- Chow, H.C., Caple, M.B., and Strouse, C.E., Polyethylene powder as a stationary phase for preparative-scale reversed-phase high-performance liquid chromatography, *J. Chromatogr.*, 151, 357, 1978.
- Closs, G.L., Katz, J.J., Pennigton, F.C., Thomas, M.R., and Strain, H.H., Nuclear magnetic resonance spectra and molecular association of chlorophylls a and b, methyl chlorophyllides, pheophytins and methylpheophorbides, *J. Am. Chem. Soc.*, 85, 3809, 1963.

- Clydesdale, F.M. and Francis, F.J., Chlorophyll changes in thermally processed spinach as influenced by enzyme conversion and pH adjustment, *Food Technol.*, 22, 793, 1968.
- Clydesdale, F.M. and Francis, F.J., Chlorophylls, in *Principles of Food Science*, Vol. 1, Fennema, O.R., Ed., Marcel Dekker, New York, 1976, p. 386.
- Clydesdale, F.M., Fleischman, D.L., and Francis, F.J., Maintenance of color in processed green vegetables, *J. Food Prod. Dev.*, 4, 127, 1970.
- Clydesdale, F.M., Lin, Y.D., and Francis, F.J., Formation of 2-pyrrolidone-5-carboxylic acid from glutamine during processing and storage of spinach puree, *J. Food Sci.*, 37, 45, 1972.
- Constantin, E., Nakatani, Y., Teller, G., Hueber, R., and Ourisson, G., Electron-impact and chemical ionization mass-spectrometry of chlorophylls, pheophytins and phaeophorbides by fast desorption on a gold support, *Bull. Soc. Chim. Fr.*, 7/8, 303, 1981.
- Craft, N.E., Wise, S.A., and Soares, J.H., Optimization of an isocratic high-performance liquid chromatographic separation of carotenoids, *J. Chromatogr.*, 589, 171, 1992.
- Dashwood, R.H., Chlorophylls as anticarcinogens, *Int. J. Oncol.*, 10, 721, 1997.
- Davies, D. and Holdsworth, E.S., The use of high pressure liquid chromatography for the identification and preparation of pigments concerned in photosynthesis, *J. Liq. Chromatogr.*, 3, 123, 1980.
- De Jong, D.W. and Woodlief, W.C., High speed, low pressure liquid chromatography of chloroplast pigments from tobacco mutants, *J. Agric. Food Chem.*, 26, 1281, 1978.
- De la Mar, R.R. and Francis, F.J., Carotenoids degradation in bleached paprika, *J. Food Sci.*, 34, 287, 1969.
- Devlin, R.M., Ed., *Fisiología Vegetal.*, Omega, Barcelona, 1975.
- Dietrich, W.C. and Neumann, H.J., Blanching brussels sprouts, *J. Food Technol.*, 19, 150, 1965.
- Dougherty, R.C., Dreifus, P.A., Sphon, J., and Katz, J.J., Hydration behavior of chlorophyll *a*: a field desorption mass spectra study, *J. Am. Chem. Soc.*, 102, 416, 1980.
- Drake, S.R., Spaid, S.E., and Thompson, J.B., The influence of blanch and freezing method on the quality of selected vegetables, *J. Food Qual.*, 4, 271, 1981.
- Eckardt, C.B., Keely, B.J., and Maxwell, J.R., Identification of chlorophyll transformation products in a lake sediment by combined liquid chromatography-mass spectrometry, *J. Chromatogr.*, 557, 271, 1991.
- Eheart, M.S. and Odland, D., Use of ammonium compounds for chlorophyll retention in frozen green vegetables, *J. Food Sci.*, 38, 202, 1973.
- Endo, Y., Thorsteinson, C.T., and Daun, J.K., Characterization of chlorophyll pigments presents in cannola seeds, meals and oils, *J. Am. Oil Chem. Soc.*, 69, 564, 1992.
- Eskins, K., Scholfield, C.R., and Dutton, H.J., High-performance liquid chromatography of plant pigments, *J. Chromatogr.*, 135, 217, 1977.
- Eskins, K. and Dutton, H.J., Sample preparation for high-performance liquid chromatography of higher plant pigments, *Anal. Chem.*, 51, 1885, 1979.
- Eskins, K. and Harris, L., High-performance liquid chromatography of etioplast pigments in red kidney bean leaves, *Photochem. Photobiol.*, 33, 131, 1981.
- Evans, N., Games, D.E., Jacson, A.H., and Matlin, S.A., Applications of high-pressure liquid chromatography and fiels desorption mass spectrometry in studies of natural porphyrins and chlorophyll derivatives, *J. Chromatogr.*, 115, 325, 1975.
- Falkowski, P.G. and Sucher, J., Rapid quantitative separation of chlorophylls and their degradation products by high-performance liquid chromatography, *J. Chromatogr.*, 213, 349, 1981.
- Fishbach, H., Microdeterminations for organically combined metals in pigment of okra, *J. Assoc. Off. Agric. Chem.*, 26, 139, 1943.

- Furuya, K., Inoue, H., and Shirai, T., Preparation and determination of Fe (II) and Fe (III) chlorophylls by reversed-phase high-performance liquid chromatography, *Anal. Sci.*, 3, 353, 1987.
- Furuya, K., Ohki, N., Inoue, H., and Shirai, T., Preparation and determination of Ni(II) chlorophylls by reversed-phase high-performance liquid chromatography, *Chromatographia*, 25, 319, 1988.
- Gandul-Rojas, B. and Mínguez-Mosquera, M.I., Chlorophyllase activity in olive fruits and its relationship with the loss of chlorophyll pigments in the fruits and oils, *J. Sci. Food Agric.*, 72, 291, 1996a.
- Gandul-Rojas, B. and Mínguez-Mosquera, M.I., Chlorophyll and carotenoids composition in virgin olive oils from various spanish olive varieties, *J. Sci. Food Agric.*, 72, 31, 1996b.
- Gandul-Rojas, B., Gallardo-Guerrero, M.L., and Mínguez-Mosquera, M.I., Identification of oxidized chlorophylls and metallochlorophyllic complexes of copper in table olives (cv. Gordal) with green staining alteration, *J. Food Prot.*, 62, 1172, 1999a.
- Gandul-Rojas, B., Roca-López-Cepero, M., Carmona-Ramón, C., and Mínguez-Mosquera, M.I., Stability of chlorophyll pigments during storage of virgin olive oil in Proceeding of the *1st International Congress on Pigments in Food Technology*, Mínguez-Mosquera, M.I., Jarén-Galán, M. and Hornero-Méndez, D., Eds., PFT, Sevilla, Spain, March 1999b.
- Gentile, J.M., Rahimi, S., Zwiesler, J., Gentile, G.J., and Ferguson, L.R., Effect of selected antimutagens on the genotoxicity of antitumor agents, *Mutat. Res.*, 402, 289, 1998.
- Ginsburg, S. and Matile, P., Identification of catabolites of chlorophyll-porphyrin in senescent rape cotyledons, *Plant Physiol.*, 102, 521, 1993.
- Ginsburg, S., Schellenberg, M., and Matile, Ph., Cleavage of chlorophyll-porphyrin. Requirement for reduced ferredoxin and oxygen, *Plant. Physiol.*, 105, 545, 1994.
- Goedheer, J.C., Visible absorption and fluorescence of chlorophyll and its aggregates in solution, in *The Chlorophylls*, Vernon, L.P. and Seeley, G.R., Eds., Academic Press, New York, 1966, p. 21.
- Goodwin, R.H., Fluorometric method for estimating small amounts of chlorophyll a, *Anal. Chem.*, 19, 789, 1947.
- Grese, R.P., Cerny, R.L., Gross, M.L., and Senge, M., Determination of structure and properties of modified chlorophylls by using fast atom bombardment combined with tandem mass spectrometry, *J. Am. Soc. Mass Spectrom.*, 1, 72, 1990.
- Grese R.P. and Gross, M.L., The role of the matrix in fast atom bombardment desorption of chlorophyll, *Int. J. Mass Spectrom. Ion Proc.*, 122, 51, 1992.
- Gross, J., Ed., *Chlorophylls*, in *Pigments in Fruits*, Academic Press, London, 1987, pp. 1–55.
- Gross, J., *Pigments in Vegetables. Chlorophylls and Carotenoids*, Van Nostrand Reinhold, New York, 1991.
- Grotemeyer, J., Bosel, U., Walter, K., and Schlag, E.W., Multiphotonionization mass spectroscopy of native chlorophylls, *J. Am. Chem. Soc.*, 108, 4233, 1986.
- Harris, P.G., Carter, J.F., Head, R.N., Harris, R.P., Eglinton, G., and Maxwell, J.R., Identification of chlorophyll transformation products in zooplankton faecal pellets and marine sediment extracts by liquid chromatography/mass spectrometry atmospheric pressure chemical ionization, *Rapid Commn. Mass Spectrom.*, 9, 1177, 1995.
- Heaton, J.W. and Marangoni, A.G., Chlorophyll degradation in processed foods and senescent plant tissues, *Trends Food Sci. Technol.*, 7, 8, 1996.
- Higashi-Okai, K., Otani, S., and Okai, Y., Potent suppressive activity of pheophytin a and b from the non-polyphenolic fraction of green tea (*Camellia sinensis*) against tumor promotion in mouse skin, *Cancer Lett.*, 129, 223, 1998.
- Holden, M., Chlorophyll bleaching by legume seeds, *J. Sci. Food Agric.*, 16, 312, 1965.

- Holden, M., Analytical methods — chlorophylls, in *Chemistry and Biochemistry of Plant Pigments*, Vol. 2, 2nd ed., Goodwin, T.W., Ed., Academy Press, New York, 1976.
- Hörtensteiner, S., Vicentini, F., and Matile, Ph., Chlorophyll breakdown in senescent leaves: enzymic cleavage of pheophorbide *a* *in vitro*, *New Phytol.*, 129, 237, 1995.
- Humphrey, A.M., Chlorophyll, *Food Chem.*, 5, 57, 1980.
- Hunt, J.E., MacFarlane, R.D., Katz, J.J., and Dougherty, R.C., High-energy fragmentation of chlorophyll *a* and its fully deuterated analogue by ^{252}Cf plasma desorption mass spectrometry, *J. Am. Chem. Soc.*, 103, 6775, 1981.
- Hynninen, P.H., Chlorophylls. IV. Preparation and purification of some derivatives of chlorophylls *a* and *b*, *Acta Chem. Scand.*, 27, 1771, 1973.
- Hyvarinen, K. and Hynninen, P.H., Liquid chromatographic separation and mass spectrometric identification of chlorophyll *b* allomers, *J. Chromatogr.*, A, 837, 107, 1999.
- Inoue, H., Furuya, K., Watanabe, K., Tanaka, K., Shirai, T., and Miyoshi, E., Preparation and determination of Cu (II) chlorophylls by reversed-phase high-performance liquid chromatography, *Anal. Sci.*, 4, 599, 1988.
- Inoue, H., Imai, M., Naemura, T., Furuya, K., and Shizuri, Y., Preparation and determination of zinc (II) chlorophylls by reversed-phase high-performance liquid chromatography, *J. Chromatogr.*, 645, 259, 1993.
- Inoue, H., Yamashita, H., Furuya, K., Nonomura, Y., Yoshioka, N., and Li, S., Determination of copper (II) chlorophyllin by reversed-phase high-performance liquid chromatography, *J. Chromatogr.*, 679, 99, 1994.
- Iriyama, K., Yoshiura, M., and Shiraki, M., Micro-method for the qualitative and quantitative analysis of photosynthetic pigments using high-performance liquid chromatography, *J. Chromatogr.*, 154, 302, 1978.
- Jackson, A.H., Structure, properties and distribution of chlorophylls, in *Chemistry and Biochemistry of Plant Pigments*, Vol. 2, 2nd ed., Goodwin, T.W., Ed., Academic Press, New York, 1976, p. 1.
- Jackson A.H., Applications of mass spectrometry in studies of porphyrins and related tetrapyrroles, *Philos. Trans. R. Soc. London Ser. A*, 293, 21, 1979.
- Jacobsen, T.R., A quantitative method for the separation of chlorophylls *a* and *b* from phytoplankton pigments by high pressure liquid chromatography, *Mar. Sci. Commun.*, 4, 33, 1978.
- Janson, T.R. and Katz, J.J., NMR spectra of diamagnetic porphyrins, in *The Porphyrins*, Vol. 4, Dolphin, D., Ed., Academic Press, New York, 1978, p. 1.
- Jeffrey, S.W., Quantitative thin-layer chromatography of chlorophylls and carotenoids from marine algae, *Biochim. Biophys. Acta*, 162, 271, 1968.
- Jones, O.T.G., The inhibition of bacteriochlorophyll biosynthesis in *Rhodospseudomonas sphaeroides* by 8-hydroxyquinoline, *Biochim. J.*, 85, 335, 1963.
- Jones, I.D., White, R.C., and Gibbs, E., Some pigment changes in cucumbers during brining and brine storage, *Food Technol.*, March, 96, 1962.
- Jones, I.D., White, R.C., and Gibbs, E., Influence of blanching or brining treatments on the formation of chlorophyllides, pheophytins, and pheophorbides in green plant tissue, *J. Food Sci.*, 28, 437, 1963.
- Jones, I.D., White, R.C., Gibbs, E., and Denard, C.D., Absorption spectra of copper and zinc complexes of pheophytins and pheophorbides, *J. Agric. Food Chem.*, 16, 80, 1968.
- Jones, I.D., Butler, L.S., Gibbs, E., and White, R.C., An evaluation of reversed phase partition for thin-layer chromatographic identification of chlorophylls and derivatives, *J. Chromatogr.*, 70, 87, 1972.
- Katz, J.J., Closs, G.L., Pennington, F.C., Thomas, M.R., and Strain, H.H., Infrared spectra, molecular weights, and molecular association of chlorophylls *a* and *b*, methyl chlorophyllides, and pheophytins in various solvents, *J. Am. Chem. Soc.*, 85, 3801, 1963.

- Katz, J.J., Dougherty, R.C., and Boucher, L.J., Infrared and nuclear magnetic resonance spectroscopy of chlorophyll, in *The Chlorophylls*, Vernon, L.P. and Seely, G.R., Eds., Academic Press, New York, 1966, p. 186.
- Katz, J.J., Chlorophyll function in photosynthesis, in *The Chemistry of Plant Pigments*, Chichester, C.O., Ed., Academic Press, New York, 1972, p. 103.
- Khachik, F., Beecher, G.R., and Whittaker, N.F., Separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography, *J. Agric. Food Chem.*, 334, 603, 1986.
- Kirk, J.T.O. and Tilney-Basset, R.A.E., *The Plastids*. 2nd ed., Elsevier, Amsterdam, 1978.
- Kostiainen, R., Hyvärinen K., and Hynninen P.H., Fast-atom bombardment mass spectra of the 132-epimers of 132-chlorophyll a, *Rapid Commun. Mass Spectrom.*, 9, 555, 1995.
- Kumar, S.S., Chaubey, R.C., Devasagayam, T.P.A., Priyadarsini, K.I., and Chauhan P.S., Inhibition of radiation-induced DNA damage in plasmid pBR322 by chlorophyllin and possible mechanism(s) of action, *Mutat. Res.*, 425, 71, 1999.
- Kuronen, P., Hyvärinen, K., Hynninen, H., and Hynninen, P.H., High-performance liquid chromatographic separation and isolation of the methanolic allomerization products of chlorophyll a, *J. Chromatogr.*, 654, 93, 1993.
- Laborde, L.F. and von Elbe, J.H., Method for improving the color of containerized green vegetables, U.S. Patent No. 5,482,727, 1996.
- Lajollo, F.M., Tannenbaum, S.R., and Labuza, T.P., Reaction at limited water concentration-chlorophyll degradation, *J. Food Sci.*, 36, 850, 1971.
- Lajollo, F.M. and Lanfer Marquez, U.M., Chlorophyll degradation in a spinach system at low and intermediate water activities, *J. Food Sci.*, 47, 1995, 1982.
- Langmeier, M., Ginsburg, S., and Matile, P., Chlorophyll breakdown in senescent leaves: demonstration of Mg-dechelataase activity, *Physiol. Plant.*, 189, 347, 1993.
- Leake, L.H. and Kirk, L.K., Method for Color Preservation in Canned Green Vegetables, U.S. Patent No. 5,114,24, 1992.
- Lee, S.M., Rhee, S.H., Yoo, J.S., and Park, K.Y., Identification of antimutagenic compounds from kale by high performance liquid chromatography and mass spectrometry, *J. Food Sci. Nutr.*, 3, 334, 1998.
- Li, S. and Inoue, H., Preparation and determination of Mn(II) and Mn(III) chlorophylls by reversed-phase high-performance liquid chromatography, *Chromatographia*, 33, 567, 1992.
- Li, S.J., Gao, S., Weng, S.F., Xu, Z.H., Inoue, H., and Wu, J.G., Spectroscopic characteristic of La(II) chlorophyll, *Prog. Nat. Sci.*, 7, 442, 1997.
- Lichtenthaler, H.K., Verbreitung und relative Konzentration der lipophilen Plastidenchinone in grünen Pflanzen, *Planta*, 81, 140, 1968.
- Lin, R.I.S., Phytochemicals and antioxidants, in *Functional Food*, Goldberg, I., Ed., Chapman & Hall, New York, 1994, p. 393.
- Livingston, R., Watson, W.F., and McArdle, J., Activation of the fluorescence of chlorophyll solutions, *J. Am. Chem. Soc.*, 71, 1542, 1949.
- Lord, C.E.C. and Tirimanna, A.S.L., A qualitative study of the carotenoid pigments of sri lanka chillies (*Capsicum annum*), *Mikrochim. Acta*, 1, 469, 1976.
- Mackinney, G. and Joslyn, M.A., The conversion of chlorophyll to pheophytin, *J. Am. Chem. Soc.*, 62, 231, 1940.
- Mantoura, R.F.C. and Llewellyn, C.A., The rapid determination of algal chlorophyll and carotenoid pigments and their breakdown products in natural waters by reverse-phase high-performance liquid chromatography, *Anal. Chim. Acta.*, 151, 297, 1983.
- McFeeters, R.F., Chichester, C.O., and Whitaker, J.R., Purification and properties of chlorophyllase from *Ailanthus altissima*, *Plant. Physiol.*, 47, 609, 1971.

- Mínguez-Mosquera, M.I. and Gallardo-Guerrero, M.L., Anomalous transformation of chloroplastic pigments in gordal variety olives during processing for table olives, *J. Food Prot.*, 58, 1241, 1995.
- Mínguez-Mosquera, M.I. and Gandul-Rojas, B., High-performance liquid chromatographic study of chlorophyll alkaline treatment, *J. Chromatogr.*, 690, 161, 1995.
- Mínguez-Mosquera, M.I. and Garrido-Fernández, J., Eliminación de compuestos lipídicos durante la fase de extracción de pigmentos cloroplásticos en aceitunas (*Olea europaea*), *Grasas Aceites*, 36, 376, 1985.
- Mínguez-Mosquera, M.I. and Garrido-Fernández, J., Chlorophyll and carotenoid presence in olive fruit (*Olea europaea*, L.), *J. Agric Food Chem.*, 37, 1, 1989.
- Mínguez-Mosquera, M.I., Garrido-Fernández, J., and Gandul-Rojas, B., Pigment changes in olives during fermentation and brine storage, *J. Agric. Food Chem.*, 37, 8, 1989a.
- Mínguez-Mosquera, M.I., Gandul-Rojas, B., and Garrido-Fernández, J., Determinación cuantitativa de clorofilidas y feoforbidas, *Grasas Aceites*, 40, 114, 1989b.
- Mínguez-Mosquera, M.I., Gandul-Rojas, B., Garrido-Fernández, J., and Gallardo-Guerrero, L., Pigments present in virgin olive oil, *J. Am. Oil Chem. Soc.*, 67, 192, 1990.
- Mínguez-Mosquera, M.I., Jarén-Galán, M., Hornero-Méndez, D., Garrido-Fernández, J., Gallardo-Guerrero, M.L., and Gandul-Rojas, B., Decoloration of vegetable oils and oleoresins with recovery of unaltered pigments, *J. Am. Oil Chem. Soc.*, 68, 809, 1991a.
- Mínguez-Mosquera, M.I., Gandul-Rojas, B., Montañó-Asquerino, A., and Garrido-Fernández, J., Determination of chlorophylls and carotenoids by HPLC during olive lactic fermentation, *J. Chromatogr.*, 585, 259, 1991b.
- Mínguez-Mosquera, M.I., Jarén Galán, M., Gandul-Rojas, B., and Fiestas Ros de Ursinos, J., Chlorophylls and carotenoids composition of the algal flora developed under immobilization conditions. Influence of support, *Biomass Bioenergy*, 1, 347, 1991c.
- Mínguez-Mosquera, M.I., Gandul-Rojas, B., and Gallardo-Guerrero, L., Rapid method of quantification of chlorophylls and carotenoids in virgin olive oil by HPLC, *J. Agric. Food Chem.*, 40, 60, 1992a.
- Mínguez-Mosquera, M.I., Jarén-Galán, M., and Gandul-Rojas, B., Pigments in the algal biomass from aerobic waste water depuration ponds, *Water, Air, and Soil Pollut.*, 65, 195, 1992b.
- Mínguez-Mosquera, M.I., Gallardo-Guerrero, L., and Gandul-Rojas, B., Characterization and separation of oxidized derivatives of pheophorbides a and b by TLC and HPLC, *J. Chromatogr.*, 633, 295, 1993.
- Mínguez-Mosquera, M.I., Gandul Rojas, B., and Mínguez-Mosquera, J., Mechanism and kinetic of the degradation of chlorophylls during the processing of green table olives, *J. Agric. Food Chem.*, 42, 1089, 1994a.
- Mínguez-Mosquera, M.I., Gandul-Rojas, B., and Gallardo-Guerrero, M.L., Measurement of chlorophyllase activity in olive fruit (*Olea europaea*), *J. Biochem.*, 116, 263, 1994b.
- Mínguez-Mosquera, M.I., Gallardo-Guerrero, M.L., Hornero-Méndez, D., and Garrido-Fernández, J., Involvement of copper and zinc ions in green staining of table olives of the variety gordal, *J. Food Prot.*, 58, 564, 1995.
- Mínguez-Mosquera, M.I., Gandul-Rojas, B., and Garrido-Fernandez, J., Preparation of Cu (II) complexes of oxidized chlorophylls and their determination by thin-layer and high-performance liquid chromatography, *J. Chromatogr.*, 731, 261, 1996.
- Moran, R. and Porath, D., Chlorophyll determination in intact tissues using N,N-dimethylformamide, *Plant. Physiol.*, 65, 478, 1980.
- Muftugil, N., Effect of different types of blanching on the color and the ascorbic acid and chlorophylls content of green beans, *J. Food Process. Preserv.*, 10, 69, 1986.

- Negishi, T., Rai, H., and Hayatsu, H., Antigenotoxic activity of natural chlorophylls, *Mutat. Res.*, 376, 97, 1997.
- Nonomura, Y., Hatano, H., Fukuda, K., and Inoue H., Preparation and determination of cobalt (II) chlorophylls by high-performance liquid chromatography, *Anal. Sci.*, 10, 117, 1994.
- Omata, T. and Murata, N., A rapid and efficient method to prepare chlorophyll *a* and *b* from leaves, *Photochem. Photobiol.*, 31, 183, 1980.
- Omata, T. and Murata, N., Preparation of chlorophyll *a*, chlorophyll *b*, and bacteriochlorophyll *a* by column chromatography with DEAE-Sepharose CL-6B and Sepharose CL-6B, *Plant Cell Physiol.*, 24, 1093, 1983.
- O'Neill, E.J. and Crener, M.S., Use of vibratory ball-mill for the extraction of plant pigments, *Analyst (London)*, 105, 625, 1980.
- Oquist, G. and Samuelsson, G., Sequential extraction of chlorophyll from chlorophyll-protein complexes in lyophilized pea thylakoids with solvents of different polarity, *Physiol. Plant.*, 50, 57, 1980.
- Perkins, H.J. and Roberts, D.W.A., Purification of chlorophylls, pheophytins and pheophorbides for specific activity determinations, *Biochim. Biophys. Acta*, 58, 486, 1962.
- Perrin, P.W. and Gaye M.M., Effects of simulated retail display and overnight storage treatments on quality maintenance in fresh broccoli, *J. Food Sci.*, 51, 146, 1986.
- Posthumus, M.A., Kistemaker, P.G., Meuzelaar, H.L.C., and Ten Noever de Braw, M.C., Laser desorption-mass-spectrometry of polar nonvolatile bio-organic molecules, *Anal. Chem.*, 50, 985, 1978.
- Rahmani, M. and Csallany, A.S., Chlorophyll and β -carotene pigments in moroccan virgin olive oils measured by high-performance liquid chromatography, *J. Am. Oil Chem. Soc.*, 68, 672, 1991.
- Rebeiz, C.A., Bazzaz, M.B., and Belanger, F., The separation of chlorophyll and pheophytins by reversed phase HPLC, *Chromatogr. Rev.*, 4, 2, 1978.
- Ruskov, P. and Malchev, E., Changes in chlorophyll of green pepper during lactic acid fermentation, *Food Sci. Technol. Abstr.*, 8J, 1048, 1971.
- Sahlberg, I. and Hynninen, P.H., Thin-layer chromatography of chlorophylls and their derivatives on sucrose layers, *J. Chromatogr.*, 291, 331, 1984.
- Salisbury, F.B. and Ross, C.W., Photosynthesis: chloroplasts and light, in *Plant Physiology* 3rd ed., Salisbury, F.B. and Ross, C.W., Eds., Wadsworth Publishing, Belmont, CA, 1985, pp. 179–194.
- Schaber, P.M., Hunt, J.E., Fries, R., and Katz, J.J., Preparation and determination of zinc (II) chlorophylls by reversed-phase high-performance liquid chromatography, *J. Chromatogr.*, 316, 25, 1984.
- Schanderl, S.H., Marsh, G.L., and Chichester, C.O., Color reversion in processed vegetables. I. Studies on regreened pea puree, *J. Food Sci.*, 30, 312, 1965.
- Scheer, H. and Katz, J.J., Nuclear magnetic resonance spectroscopy of porphyrins and metalloporphyrins, in *Porphyrins and Metalloporphyrins*, 2nd ed., Smith, K.M., Ed., Elsevier, New York, 1975.
- Scheer, H., Chlorophylls, in *CRC Handbook of Chromatography*, Vol. 1, Kost, H.P., Ed., CRC Press, Boca Raton, FL, 1988.
- Schellenberg, M., Matile, Ph., and Thomas, H., Production of a presumptive chlorophyll catabolite *in vitro*: requirement of reduced ferredoxin, *Planta*, 191, 417, 1993.
- Schick, G.A. and Bocian, D.F., Resonance Raman studies of hydroporphyrins and chlorophylls, *Biochim. Biophys. Acta*, 895, 127, 1987.
- Schoch, S., Lempert, U., Wieschhoff, H., and Scheer, H., High-performance liquid chromatography of tetrapyrrole pigments, *J. Chromatogr.*, 157, 3567, 1978.

- Schwartz, S., Berg, M.H., Bossenmainer, I., and Dismore, H., Determination of porphyrin in biological material, in *Methods of Biochemical Analysis*, Vol. 3, Glick, D., Ed., Interscience Publishers, New York, 1957, p. 221.
- Schwartz, S.J., Woo, S.L., and von Elbe, J.H., High performance liquid chromatography of chlorophylls and their derivatives in fresh and processed spinach, *J. Agric. Food Chem.*, 29, 533, 1981.
- Schwartz, S.J., von Elbe, J.H., and Lindsay, R.C., Influence of processing on the pigmentation of wild rice grain, *J. Agric. Food Chem.*, 31, 349, 1983.
- Schwartz, S.J. and von Elbe, J.H., Kinetics of chlorophyll degradation to pyropheophytin in vegetables, *J. Food Sci.*, 48, 1303, 1983.
- Schwartz, S.J., High performance liquid chromatography of zinc and copper pheophytins, *J. Liq. Chromatogr.*, 7, 1673, 1984.
- Schwartz, S.J. and Lorenzo, T.V., Chlorophyll stability during aseptic processing and storage, Annual Institute of Food Technology Meeting, Chicago, IL, June 1989.
- Schwartz S.J. and Lorenzo, T., Chlorophylls in food. Crit. Rev., *Food Sci. Nutr.*, 29, 1, 1990.
- Segner, W.P., Ragusa, T.J., Nank, W.K., and Hoyle, W.C., Process for the preservation of green color in canned vegetables, U.S. Patent No. 4,473,591, September 25, 1984.
- Šesták, Z., Paper chromatography of chloroplast pigments, *J. Chromatogr.*, 1, 293, 1958.
- Šesták, Z., Paper chromatography of chloroplast pigments (chlorophylls and carotenoids). Part 3, *Photosynthetica*, 14, 239, 1980.
- Sherma, J. and Latta, M., Reversed phase thin-layer chromatography of chloroplast pigments on chemically bonded C₁₈ plates, *J. Chromatogr.*, 154, 73, 1978.
- Shewfelt, R.L., Batal, K.M., and Heaton, E.K., Broccoli storage: effect of N⁶-benzyladenine, packaging and icing on color of fresh broccoli, *J. Food Sci.*, 48, 1594, 1983.
- Shioi, Y., Fukae, R., and Sasa, T., Chlorophyll analysis by high-performance liquid chromatography, *Biochim. Biophys. Acta*, 722, 72, 1983.
- Shioi, Y., Michio, D., and Sasa, T., Separation of non-esterified chlorophylls by ion-suppression high-performance liquid chromatography, *J. Chromatogr.*, 298, 141, 1984.
- Shioi, Y., Analytical chromatography of chlorophylls, in *Chlorophylls*, Scheer, H., Ed., CRC Press, Boca Raton, FL, 1991.
- Shoaf, W.T., Rapid method for the separation of chlorophylls a and b by high-pressure liquid chromatography, *J. Chromatogr.*, 152, 247, 1978.
- Sievers, G. and Hynninen, P.H., Thin-layer chromatography of chlorophylls and their derivatives on cellulose layers, *J. Chromatogr.*, 134, 359, 1977.
- Simpson, K.L., Chemical changes in natural food pigments, in *Chemical Changes in Food During Processing*, Richardson, T. and Finley, J.W., Eds., AVI Publishing, Westport, CT, 1985.
- Simpson, K.L., Lee, T.C., Rodriguez, D.B., and Chichester, C.O., Metabolism in senescent and stores tissues, in *Chemistry and Biochemistry of Plant Pigments*, Vol. 1, Goodwin, T.W., Ed., Academic Press, London, 1976, p. 780.
- Sistrunk, W.A., Mahon, M.K., and Freeman, D.W., Relationship of processing methodology to quality attributes and nutritional value of canned spinach, *Hortic. Sci.*, 12, 59, 1977.
- Smith, J.H.C. and Benítez, A., Chlorophylls: analysis in plant materials, in *Modern Methods of Plant Analysis*, Vol. 4, Paech, K. and Tracey, M.V., Eds., Springer-Verlag, New York, 1955, p. 142.
- Stainer, R.Y., Duodoroff, M., and Alderberg, E.A., *El Mundo de los Microbios*, Aguilar, S.A. Madrid, 1965.
- Stewart, J.K. and Uota, M., Carbon dioxide injury and market quality of lettuce held in controlled atmosphere, *J. Am. Soc. Hortic. Sci.*, 96, 27, 1971.

- Stobart, A.K., McLaren, I., and Thomas, D.R., Chlorophylls and carotenoids of colourless callus, green callus and leaves of *Kalanchoe crenata*, *Phytochemistry*, 6, 1467, 1967.
- Strain, H.H., Oxidation and isomerization reactions of the chlorophylls in killed leaves, *J. Agric. Food Chem.*, 2, 1122, 1954.
- Strain, H.H. and Svec, W.A., Extraction, separation, estimation and isolation of the chlorophylls, in *The Chlorophylls*, Vernon, L.P. and Seely, G.R., Eds., Academic Press, New York, 1966.
- Strain, H.H., Sherma, J., and Grandolfo, M., Alteration of chloroplast pigments by chromatography with siliceous adsorbents, *Anal. Chem.*, 39, 926, 1967.
- Strain, H.H. and Svec, W.A., Some procedures for the chromatography of the fat-soluble chloroplast pigments, *Adv. Chromatogr.*, 8, 118, 1969.
- Strain, H.H., Cope, B.T., and Svec, W.A., Analytical procedures for the isolation, identification, estimation and investigation of the chlorophylls, *Methods Enzymol.*, 23, 452, 1971.
- Strain, H.H. and Sherma, J., Investigations of the chloroplast pigments of higher plants, green algae and brown algae and their influence upon the invention, modifications, and applications of Tsweet's chromatographic method, *J. Chromatogr.*, 73, 371, 1972.
- Stransky, H., Die quantitative Bestimmung von Chloroplastenpigmenten im Picomol-Bereich mit Hilfe einer isochratischen HPLC-Methode, *Z. Naturforsch.*, 33, 836, 1978.
- Strasburger, E., *Tratado de Botánica*, 6ª edición española, Manuel Marín y Cia, Barcelona, 1974.
- Suzuki, N., Saitoh, K., and Adachi, K., Reversed-phase high-performance thin-layer chromatography and column liquid chromatography of chlorophylls and their derivatives, *J. Chromatogr.*, 408, 181, 1987.
- Svec, W.A., The isolation, preparation, characterization, and estimation of the chlorophylls and the bacteriochlorophylls, in *The Porphyrins*, Vol. 5, Physical Chemistry, Part C, Dolphin, D., Ed., Academic Press, New York, 1978.
- Sweeney, J.P. and Martin, M.E., Determination of chlorophyll and pheophytin in broccoli heated by various procedures, *Food Res.*, 23, 635, 1958.
- Sweeney, J.P. and Martin, M.E., Stability of chlorophyll in vegetables as affected by pH, *Food Technol.*, 15, 263, 1961.
- Tabet, J.C., Jablonski, M., Cotter, R.J., and Hunt, J.E., Time resolved laser desorption. III. The metastable decomposition of chlorophyll *a* and some derivatives, *Int. J. Mass Spectrom. Ion Phys.*, 65, 105, 1985.
- Takeda, Y., Konuma, H., Uchiyama, S., and Saito, Y., Microbial formation of pheophorbide *a* and pyropheophorbide *a* in *Takana* during brining and fermentation, *J. Food Hyg. Soc. Jpn.*, 30, 228, 1990.
- Tan, C.T. and Francis, F.J., Effect of processing temperature on pigments and color of spinach, *J. Food Sci.*, 27, 232, 1962.
- Te, C., Gentile, J.M., Baguley, B.C., Pearson, A.E., Gregory, T., and Ferguson, L.R., *In vivo* effects of chlorophyllin on the antitumor agent cyclophosphamide, *Int. J. Cancer*, 70, 84, 1997.
- Teng, S.S. and Chen, B.H., Formation of pyrochlorophylls and their derivatives in spinach leaves during heating, *Food Chem.*, 65, 367, 1999.
- Terpstra, W. and Lambers, J.W.J., Interactions between chlorophyllase, chlorophyll *a*, plant lipids and Mg^{2+} , *Biochim. Biophys. Acta*, 746, 23, 1983.
- Tonucci, L.H. and von Elbe, J.H., Kinetics of the formation of zinc complexes of chlorophyll derivatives, *J. Agric. Food Chem.*, 40, 2341, 1992.
- Torsi, G., Chiavari, G., and Lippolis, M.T., Quantitation without calibration curves using HPLC with nondestructive detectors, *LC-GC INTL*, 5, 37, 1992.

- Tswett, M., Adsorptionsanalyse and chromatographische methods, Anwendung auf die Chemie des Chlorophylls, *Ber. Dtsch. Bot. Ges.*, 24, 384, 1906.
- Usuki, R., Suzuki, T., Endo, Y., and Kaneda, T., Residual amounts of chlorophylls and pheophytins in refined edible oils, *J. Am. Oil Chem. Soc.*, 61, 785, 1984.
- Van Breemen, R.B., Canjura, F.L., and Schwartz, S.J., Identification of chlorophyll derivatives by mass spectrometry, *J. Agric. Food Chem.*, 39, 1452, 1991a.
- Van Breemen, R.B., Canjura, F.L., and Schwartz, S.J., High-performance liquid chromatography-continuous-flow fast atom bombardment mass spectrometry of chlorophyll derivatives, *J. Chromatogr.*, 542, 373, 1991b.
- Van Buren, J.P., Extraction of chlorophylls a and b from different binding sites on thylakoid chlorophyll-proteins, *J. Agric. Food Chem.*, 33, 204, 1985.
- Vernon, L.P. and Seely, G.R., Eds., *The Chlorophylls*, Academic Press, New York, 1966.
- Verzegnassi, L., Riffe-Chalard, C., Kloeti, W., and Gulacar, F.O., Analysis of tetrapyrrolic pigments and derivatives in sediments by high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry, *J. Anal. Chem.*, 364, 249, 1999.
- Virgin, H.I., Protochlorophyll formation and greening in etiolated barley leaves, *Physiol. Plant.*, 8, 630, 1955.
- Virgin, H.I., Action spectrum for the elimination of the lag phase in chlorophyll formation in previously dark grown leaves of wheat, *Physiol. Plant.*, 14, 439, 1961.
- von Elbe, J.H., Huang, A.S., Attoe, E.L., and Nank, W.K., Pigment composition and color of conventional and Veri-Green® canned beans, *J. Agric. Food Chem.*, 34, 52, 1986.
- Walker, G.C., Color deterioration in frozen French beans (*Phaseolus vulgaris*). I, *J. Food Sci.*, 29, 383, 1964.
- Wang, S.S., Haard, N.F., and Dimarco, G.R., Chlorophyll degradation during controlled atmosphere storage of asparagus, *J. Food Sci.*, 36, 657, 1971.
- Ward, K., Scarth, R., Daun, J.K., and Thorsteinson, C.T., Effects of processing and storage on chlorophyll derivatives in commercially extracted canola oil, *J. Am. Oil Chem. Soc.*, 71, 811, 1994.
- Watanabe, T., Hongu, A., Honda, K., Nakazato, M., Konno, M., and Saithoh, S., Preparation of chlorophylls and pheophytins by isocratic liquid chromatography, *Anal. Chem.*, 56, 251, 1984.
- Weemaes, C., Ooms, V., Indrawati-Ludikhuyze, L., van den Broeck, I., van Loevy, A., and Hendrickx, M., Pressure-temperature degradation of color in broccoli juice, *J. Food Sci.*, 64, 504, 1999.
- White, R.C., Jones, I.D., and Gibbs, E., Determination of chlorophylls, chlorophyllides, pheophytins and pheophorbides in plant material, *J. Food Sci.*, 28, 431, 1963.
- White, R.C., Jones, I.D., Gibbs, E., and Butler, L.S., Fluorometric estimation of chlorophylls, chlorophyllides, pheophytins and pheophorbides in mixtures, *J. Agric. Food Chem.*, 20, 773, 1972.
- Willstätter, R. and Stoll, A., *Investigations on Chlorophyll* (English translation by Schertz, F.M. and Merz, R.A.), The Science Press Printing, Lancaster, PA, 1928, p. 385.
- Wright, S.W. and Shearer, J.D., Rapid extraction and high-performance liquid chromatography of chlorophylls and carotenoids from marine phytoplankton, *J. Chromatogr.*, 294, 281, 1984.
- Yang, H., Li, M., Zhang, W., Zhao, H., and Zhang, Z., Characteristics of photosensitization of pheophorbide a in liposomal media, *Sci. China, Ser. C: Life Sci.*, 42, 471, 1999.
- Yasuda K., Tadano, K., Ushiyama, H., Ogawa, H., Kawai, Y., and Nishima T., Investigation to find and indicator substance for the analysis of sodium copper chlorophyllin in foods, *J. Food Hyg. Soc. Jpn.*, 36, 710, 1995.

- Yoshiura, M., Iriyama, K., and Shiraki, M., High-performance liquid chromatography of chlorophylls and some of their derivatives, *Chem. Lett.*, 281, 1978.
- Yost, R.W., Ettre, L.S., and Conlon, R.D., *Introducción a la Cromatografía Líquida Práctica* (Spanish translation by Sanchez, F.), The Perkin-Elmer Corp., Norwalk, CT, 1980.
- Ziegler, R., Blaheta, A., Guha, N., and Schönege, B., Enzymatic formation of pheophorbide and piropheophorbide during chlorophyll degradation in a mutant of *Chlorella fusca* Shihira et Kraus, *J. Plant Physiol.*, 132, 327, 1988.
- Zissis, K.D., Dunkerley, S., and Brereton, R.G., Chemometric techniques for exploring complex chromatograms: application of diode array detection high performance liquid chromatography electrospray ionization mass spectrometry to chlorophyll a allomers, *Analyst*, 124, 971, 1999.
- Zonneveld, H., Klop, W., and Gorin, N., Analysis of unboiled and boiled beans for chlorophylls and pheophytins by thin-layer chromatography and fluorimetry comparison with spectrometry, *Z. Lebensm. Unters. Forsch.*, 178, 20, 1984.

5 Anthocyanins

Gary Takeoka and Lan Dao

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5.1 STRUCTURE AND CHEMISTRY

Anthocyanins, the largest group of water-soluble pigments in the plant kingdom, are responsible for most of the red, blue, and purple colors of fruits, vegetables, flowers, and other plant tissues (Harborne, 1967). The anthocyanins are all based chemically on a single aromatic structure, cyanidin, and all are derived from this compound by the addition or subtraction of hydroxyl groups, by the degree of methylation of these hydroxyl groups, and by the nature and number of sugars and their position on the aglycon (Harborne, 1998). Additionally, sugars can be esterified with aliphatic or aromatic acids leading to mono- and polyacylated anthocyanins. There are six common anthocyanins whose structures are shown in Figure 5.1. With a few exceptions anthocyanins are always glycosylated at C-3. The most common sugar is

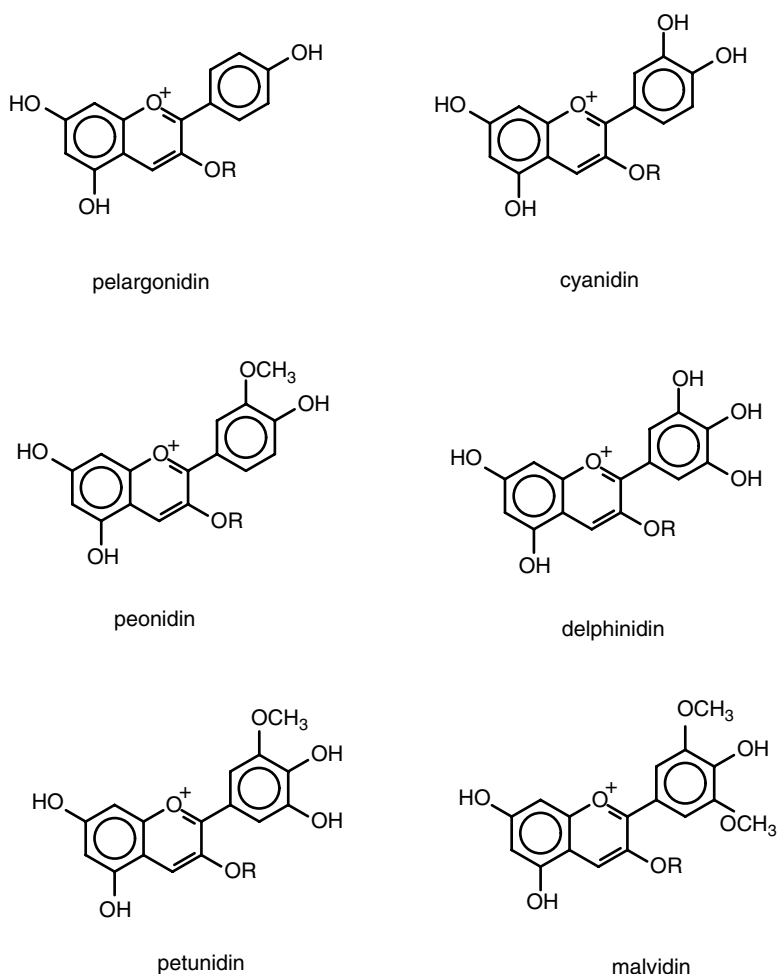


FIGURE 5.1 Structures of six common anthocyanins. R, sugar moiety. This is often glucose, but it may also be galactose, rhamnose, xylose, or arabinose.

glucose, but arabinose, galactose, rhamnose, or xylose may also be present. Many di- and trisaccharides are known to occur with combinations of the previously mentioned sugars. Besides the C-3 position, other sugars can also be attached at any one of the hydroxyls at C-5, C-7, C-3', C-5', and even C-4' (Brouillard, 1988). Sugars may be acylated with acetic, malonic, malic, oxalic, succinic, *p*-hydroxybenzoic, or hydroxycinnamic (*p*-coumaric, caffeic, ferulic, or sinapic) acids, with acylation most commonly occurring with the sugar moiety at the C-3 position. Acylation with aliphatic dicarboxylic acids such as malic, malonic, oxalic, and succinic acids renders the cationic anthocyanin a zwitterion that allows the distinction between these pigments and other anthocyanins by paper electrophoresis in a weakly acidic buffer (Harborne and Grayer, 1988). Another important feature of anthocyanins acylated with aliphatic acids is the labile nature of the ester bond. When malonated anthocyanins are subjected

to standard extraction procedures using methanol acidified with 0.1 to 1.0% HCl, there is an intermediate methyl ester formation at the free carboxyl group, while the main reaction is the loss of the malonyl group in a short time (Harborne, 1988).

An important distinction between anthocyanins and other flavonoids is not only that they are red, orange, violet, purple, or blue while most flavones and flavonols are yellow, but also that they give rise in aqueous solution to reactions such as proton transfer, isomerization, and tautomerization (Brouillard, 1988). Under the same conditions, the colorless flavonoids do not react and thus appear in only one chemical state. The structural transformations of anthocyanins in aqueous solution have been summarized by Brouillard (1988) (Figure 5.2). At pHs below 2, anthocyanins exist primarily in the form of the red (glycosylated at C-3) flavylium cations (AH^+) (Figure 5.2, structure 1). Solvation of a flavylium salt in a slightly acidic or neutral aqueous solution results in the immediate formation of the neutral (Figure 5.2, structures 2 to 4) and/or ionized quinonoidal bases (Figure 5.2, structures 5 to 7). However, the common 3-glycosides and 3,5-diglycosides change more or less rapidly to the more stable carbinol (Figure 5.2, structure 8) and chalcone pseudobases (Figure 5.2, structure 9). Rapid and almost complete hydration of the flavylium cation (AH^+) occurs almost exclusively at the 2-position to give the colorless carbinol pseudobase at pH values ranging from 3 to 6. This, in turn, can equilibrate, at a slower rate, to an open form, the chalcone pseudobase, which is also colorless.

Anthocyanins help to attract animals, leading to seed dispersal and pollination (Strack and Wray, 1994). There is evidence that anthocyanins may play a role in protecting plants against ultraviolet (UV)-induced damage (Bohm, 1998). They may also be beneficial to human health due to their possible role as dietary antioxidants and antiinflammatory agents (Tsuda et al., 1994; Tsuda, Horio, and Osawa, 1998; Wang et al., 1997; Wang et al., 1999).

An impressive compilation of the qualitative and quantitative composition of anthocyanins in a wide variety of fruits, cereal grains, legumes, and vegetables was produced by Mazza and Miniati (1993).

Analytical methods for the isolation, separation, and characterization of anthocyanins have been described (Jackman, Yada, and Tung, 1987; Harborne, 1998; Markham, 1982). A comprehensive and highly recommended source for anyone involved in anthocyanin analysis is that of Strack and Wray (1989). Further details of flavonoid chemistry can be found in volumes of *The Flavonoids* (Harborne, Mabry, and Mabry, 1975; Harborne and Mabry, 1982; Harborne, 1988, 1994).

Andersen and co-workers at the University of Bergen in Norway have examined the anthocyanin composition of many products; their generalized analytical approach is shown in Figure 5.3. The material of interest is extracted with methanol containing 1% trifluoroacetic acid (TFA). The extract is partitioned against ethyl acetate to remove interfering phenolic acids and flavonoids. The methanolic extract is applied to an Amberlite XAD-7 column. The column is washed with water, and the anthocyanins are eluted with 50% aqueous methanol and 100% methanol (both containing 0.1 to 0.5% TFA). The sample is then fractionated on a Sephadex LH-20 gel filtration column eluting with a MeOH–water–TFA mixture. The homogeneity of the separated anthocyanins is checked by thin-layer chromatography (TLC) and analytical high-performance liquid chromatography (HPLC). If further purification is required,

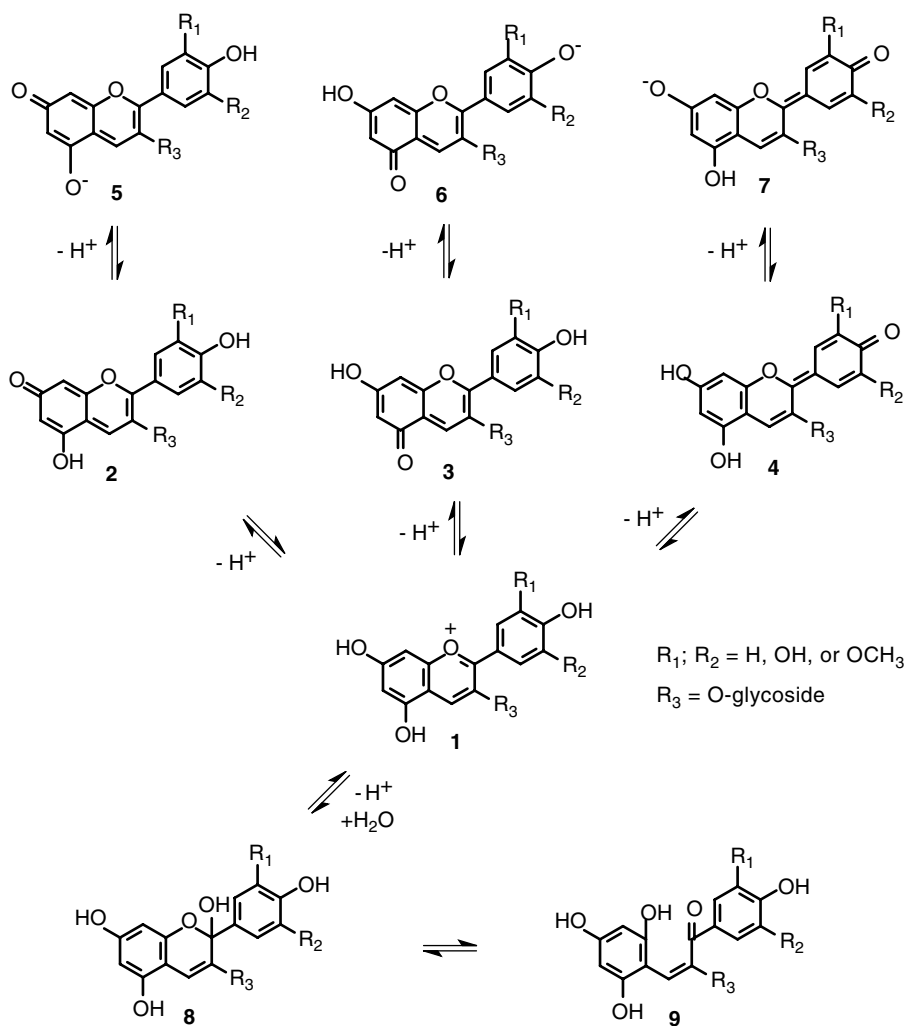


FIGURE 5.2 Structural transformations of anthocyanins in aqueous solution at varying pH: 1, flavylium cation (red to orange color); 2 to 4, neutral quinonoidal bases (purple to violet color); 5 to 7, ionized quinonoidal bases (blue color); 8, carbinol pseudobase (colorless); and 9, chalcone pseudobase (colorless). (Adapted from Brouillard, 1988.)

preparative HPLC on a reversed-phase C_{18} column is used. Characterization of purified anthocyanins is achieved by ^1H and ^{13}C NMR spectroscopy. Further details of each step are provided in the following sections.

5.1.1 AUTHENTIC STANDARDS

Delphinidin 3-glucoside and cyanidin 3-glucoside can be isolated from black currant (*Ribes nigrum*), while whortleberry (*Vaccinium myrtillus*) and strawberry (*Fragaria*

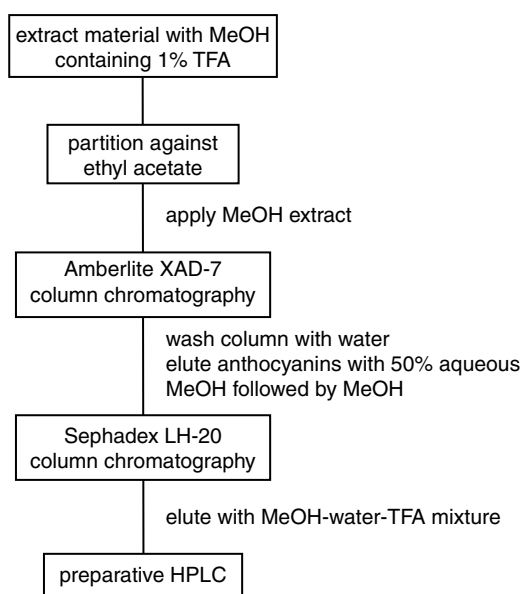


FIGURE 5.3 Generalized scheme for the isolation and purification of anthocyanins by Andersen and co-workers (University of Bergen, Norway).

ananassa) are sources of petunidin 3-glucoside and pelargonidin 3-glucoside, respectively (Kidøy et al., 1997). A limited variety of anthocyanins is available from Indofine Chemical Company (Somerville, NJ) and Extrasynthese S.A. (Genay Cedex, France), while a more extensive selection is available from Polyphenols AS (Sandnes, Norway; www.polyphenols.com).

5.2 ISOLATION

5.2.1 EXTRACTION

Due to their instability in neutral or basic solutions anthocyanins have been most commonly extracted with methanol or ethanol containing HCl (ca. 0.1 to 1%). Labile acylated anthocyanins, particularly those containing aliphatic acids, i.e., malonic acid, should be extracted with weak acids such as acetic (Gläzgen et al., 1992a; Harborne and Boardley, 1985; Markham, 1996), tartaric (Philip, 1974), or citric (Fossen and Andersen, 1997a; Main, Clydesdale, and Francis, 1978; Strack et al., 1986). Researchers performing chemotaxonomic studies have observed that the extraction of some acylated anthocyanins under acidic conditions may result in their partial or total hydrolysis (Van Sumere et al., 1985; Van Wyck and Winter, 1994). Similarly, Revilla, Ryan, and Martin-Ortega (1998) have shown that the use of solvent containing up to 1% of 12 *N* HCl caused the partial hydrolysis of some acylated anthocyanins during extraction. Artifact formation has also been observed using methanol containing 2% formic acid as the extracting solvent (Bakker and Timberlake, 1985). It is recommended that extraction protocols using organic and

mineral acids be tested against extraction procedures that use neutral solvents to verify that anthocyanins are not hydrolyzed during the extraction step. TFA (bp 72.4°C) has also been used and has the advantage that it is easily removed by evaporation under reduced pressure. Kondo et al. (1985) employed 3% aqueous TFA to isolate malonated pigments from *Monarda didyma*.

Giusti and co-workers (Giusti and Wrolstad, 1996; Giusti et al., 1998) have developed and used a novel method to extract anthocyanins from various foods. The method involves powdering the sample with liquid nitrogen, followed by blending with one volume of acetone. The sample is then filtered on a Buchner funnel using Whatman No. 1 paper. The filter cake residue is reextracted with aqueous acetone (30:70, v/v) until a clear solution is obtained. The combined filtrates are shaken in a separatory funnel with chloroform (acetone–chloroform, 1:2, v/v) and stored overnight at 1°C. The upper aqueous layer is collected, and residual acetone is removed by rotary evaporation. The aqueous extract is then made up to a known volume with distilled water. This method prevents hydrolysis of acyl substituents that may occur when using methanolic HCl.

5.3 METHODS OF SEPARATION AND PURIFICATION

5.3.1 PAPER CHROMATOGRAPHY

Paper chromatography (PC) offers the advantages of being inexpensive and able to work with crude extracts. Additionally, the R_f values on paper are more reproducible than by TLC, and identification (at least tentative) can often be achieved by comparison with published values of known standards (Harborne, 1967). However, since R_f values can vary depending on a number of factors such as temperature, composition of the developing solvent, and amount of material spotted, it is preferable to run standards along with the sample. It is quite convenient to carry out the separations on filter paper, typically Whatman No. 1. In preparative work, Whatman No. 3 filter paper has been widely used for the separation of individual anthocyanins. Descending PC is a useful method since the developing solvent can be easily overrun if desired (Harborne, 1998). The most popular solvent system consists of a mixture of *n*-butanol–acetic acid–water (4:1:5; BAW). This mixture exists as a two-phase system that is prepared in a separatory funnel. The upper phase is used to develop the paper, while the lower phase is used to saturate the atmosphere in the chromatography chamber. The Forestal solvent consisting of acetic acid–concentrated HCl–water (30:3:10) has also been widely used. It was originally developed for the separation of anthocyanidins (Bate-Smith, 1954) for which it gives efficient separations. It possesses the additional advantage of providing increased anthocyanidin stability compared to the previously mentioned system. Two other systems that have been used in anthocyanin analysis include *n*-butanol-2 *M* HCl (1:1, top layer; BuHCl) and 1% HCl [concentrated HCl–H₂O (3:97)]. In general, as the number of sugars units present increases, anthocyanins have decreasing R_f values in BAW and BuHCl and increasing values in 1% HCl. In contrast, the addition of acyl groups results in a decrease in R_f values in 1% HCl and increasing values in BAW and BuHCl (Harborne, 1967). Spot color provides information regarding the identity of anthocyanin

TABLE 5.1
 R_f Values of Common Anthocyanins and Anthocyanidins
by Paper Chromatography^a

Constituent	R_f values ($\times 100$)			
	BAW	BuHCl	1% HCl	Forestal
Pelargonidin 3-glucoside	44	38	14	
Cyanidin 3-glucoside	38	25	17	
Peonidin 3-glucoside	41	30	09	
Delphinidin 3-glucoside	26	11	03	
Petunidin 3-glucoside	35	14	04	
Malvidin 3-glucoside	38	15	06	
Pelargonidin	80			68
Cyanidin	68			49
Peonidin	71			63
Delphinidin	42			32
Petunidin	52			46
Malvidin	58			60

^a Data from Harborne (1967). Solvents are BAW, *n*-butanol–acetic acid–water (4:1:5, upper layer); BuHCl, *n*-butanol–HCl (1:1, upper layer); 1% HCl, water–concentrated HCl (97:3), and Forestal, acetic acid–concentrated HCl–water (30:3:10).

glycosides since pelargonidin glycosides are orange, cyanidin and peonidin glycosides are magenta, and delphinidin glycosides are mauve (Harborne, 1998). Examination under UV light is useful since certain anthocyanins, i.e., the 3,5-diglycosides of pelargonidin, peonidin, and malvidin, are distinguished from the 3-glycosides by their distinct fluorescence (Strack and Wray, 1989). However, Ribéreau-Gayon (1972) has noted that diglycosides of other anthocyanidins also show weak fluorescence on strongly acid chromatograms. Table 5.1 lists the R_f values of some common anthocyanins and their aglycons.

5.3.2 THIN-LAYER CHROMATOGRAPHY

TLC has several advantages over PC. It is more rapid, gives better resolution, has greater sensitivity, and is more versatile in that a variety of adsorbents may be used. A useful mixture for the separation of anthocyanidins on silica gel is ethyl acetate–formic acid–2 *M* HCl (85:6:9) (Harborne, 1967). The use of standard-grade silica gel is recommended since the trace metals present aid the separation of peonidin and malvidin from cyanidin and delphinidin (Harborne, 1998). Preparative TLC of anthocyanins using 20 $\times$ 20 cm chromatoplates with 1-mm layers of a mixture of two thirds silica gel (adsorbosil-2) and one third cellulose powder (MN-300, gypsum free) was described by Asen (1965). He employed the following solvent systems to purify milligram quantities of anthocyanins from plant tissues: *n*-butanol–water (1:1), water–HCl–formic acid (8:4:1), 1% HCl, and acetone–0.5 *N* HCl (1:3). Table 5.2 lists R_f values of some common anthocyanidins on microcrystalline

TABLE 5.2
R_f Values of Common Anthocyanidins
by Thin-Layer Chromatography^a

Constituent	R _f values (×100)	
	BAW	Forestal
Pelargonidin	82	61
Cyanidin	68	45
Peonidin	71	63
Delphinidin	42	30
Petunidin	52	43
Malvidin	56	58

^a Data on microcrystalline cellulose from Strack and Wray (1989). Solvents are BAW, *n*-butanol–acetic acid–water (4:1:5, upper layer) and Forestal, acetic acid–concentrated HCl–water (30:3:10).

cellulose (Strack and Wray, 1989). The simultaneous analysis of anthocyanidins and anthocyanins on cellulose layers using a solvent system of concentrated HCl–formic acid–water (24.9:23.7:51.4) has been demonstrated by Andersen and Francis (1985).

5.3.3 COLUMN CHROMATOGRAPHY

Column chromatography has long been an important tool in isolating natural products. Crude extracts are typically subjected to column chromatography before applying HPLC. Two widely used packings used to fractionate and isolate anthocyanins are Amberlite XAD-7 and Sephadex LH-20. The typical procedure is straightforward. A crude extract is loaded onto the top of a column of adsorbent, and compounds are eluted by varying the polarity of the solvent.

5.3.3.1 Amberlite XAD-7 Column Chromatography

Amberlite XAD-7 is a nonionic, moderately polar, acrylic resin that has been used to purify anthocyanins. Lacking ionic groups, it presumably separates compounds through hydrophobic and polar interactions. Andersen (1988) employed Amberlite XAD-7 column chromatography (18 × 2.6 cm) as a fractionation step (to eliminate polar nonphenolic compounds) in his elucidation of anthocyanins from *Dacrydium dacrydioides*. After extracts had been loaded onto the column, it was washed with 2 l of H₂O followed by 300 ml each of 50% aqueous MeOH and anhydrous MeOH (both containing 0.5% CF₃COOH) to elute the anthocyanins. In their investigation of anthocyanins from the flowers of *Crocus*, Nørbæk and Kondo (1998) applied extracts onto an Amberlite XAD-7 column, first washing with 0.5% TFA and then eluting anthocyanins stepwise with 4 to 20% aqueous CH₃CN containing 0.5% TFA. Catalano, Fossen, and Andersen (1998) applied a concentrated methanolic extract of flowers of *Vicia villosa* [first purified by partition (three times) against 0.3 l of

EtOAc] to an Amberlite XAD-7 column. The anthocyanins were partially purified by washing with about 3 l of water before being eluted from the resin with about 1 l of methanol–acetic acid (19:1, v/v).

5.3.3.2 Sephadex Gel Column Chromatography

Sephadex is a cross-linked dextran gel with a narrow molecular weight range. A particularly useful material in anthocyanin purification is Sephadex LH-20 (Saito et al., 1985). Sephadex LH-20 is produced by treating Sephadex G-25 with propylene oxide which replaces each hydroxyl hydrogen in the polymer with a 2-propanol group. This treatment results in a gel with increased hydrophobicity. Due to its dual lipophilic and hydrophilic nature, Sephadex LH-20 swells in a range of solvents from weak and medium polarity to strongly polar (Henke, 1995). This material, designed to be used with organic solvents and water–solvent mixtures, possesses an exclusion limit (at maximum swelling) of 4000 (Henke, 1995). Column chromatography on this packing has been used to fractionate crude extracts and has additionally been useful for the purification of individual anthocyanins (Andersen, 1988). Andersen and co-workers (Cabrita and Andersen, 1999; Fossen and Andersen, 1997b; Torskangerpoll, Fossen, and Andersen, 1999) have used both isocratic [MeOH–H₂O–TFA (49.5:50:0.5), MeOH–acetic acid–H₂O (10:1:10), or H₂O–MeOH–TFA (80:19.6:0.4, v/v/v)] and step elution [20 to 60% MeOH–H₂O (0.1% TFA)] on Sephadex LH-20 columns (100 × 5.0 cm or 100 × 1.0 cm) to purify anthocyanins. Bakker and Timberlake (1997) used Sephadex LH-20 column chromatography as their first fractionation step in their isolation and purification of anthocyanins from red wines. Wine (concentrated by rotary evaporation) was applied to a 60 × 5 cm column packed with Sephadex LH-20. The column was eluted with up to 2500 ml of aqueous 3% formic acid. Though this packing material is moderately expensive, it can be used repeatedly.

5.3.4 SOLID-PHASE EXTRACTION

Solid-phase extraction (SPE) is one of the most commonly used sample preparation techniques in liquid chromatography. Small disposable cartridges packed with silica gel, alumina, ion-exchange resins, C₈ and C₁₈ silica-based material, etc. (sorbent mass typically ranging from 10 mg to 20 g) are used to isolate the compounds of interest. As a rule, the sample components to be determined or isolated are retained quantitatively on the SPE cartridge while interfering species are washed from the cartridge. Finally, the retained sample components are eluted from the cartridge with an appropriate solvent.

SPE cartridges provide a simple and convenient means of isolating anthocyanins. Kraemer-Schafhalter, Fuchs, and Pfannhauser (1998) compared the efficiency of 16 solid-phase materials in purifying anthocyanins from *Aronia melanocarpa* var. Nero. They found that reversed-phase silica gels and macroporous nonionic acrylic polymer adsorbents such as Serolit PAD IV and Amberlite XAD-7 were the most effective in separating the anthocyanins from the sugars. Anthocyanins are typically loaded onto C₁₈ cartridges which have been previously activated by washing with methanol followed by 0.01% aqueous HCl (Hong and Wrolstad, 1990b). The cartridges are

then treated with two volumes of 0.01% aqueous HCl to remove sugars, acids, and other water-soluble compounds. Less polar phenolics such as phenolic acids and flavonoids are removed by washing with two volumes of ethyl acetate (Oszmianski and Lee, 1990). This step has been shown to be an important cleanup procedure that produces mass spectra with very few interferences from other compounds (Giusti et al., 1999a). Anthocyanins are finally eluted with methanol containing 0.01% HCl.

SPE cartridges can also be used for the stabilization of anthocyanidins. Anthocyanidins (the aglycons of anthocyanins) have limited solubility in water, are rapidly destroyed by alkali, and are very unstable compared to their corresponding glycosides (anthocyanins). The half-life of a typical anthocyanin, i.e., cyanidin 3-glucoside, is about 65 days at room temperature in 0.01 *M* citric acid, pH 2.8. In contrast, the corresponding aglycon, cyanidin, has a half-life of only 12 h in the same solution (Iacobucci and Sweeney, 1983). Ohta, Akuta, and Osajima (1980) have also shown that peonidin and malvidin are much less stable than their corresponding 3-glucosides at pH values of 2.5 and 4.5. Due to the limited commercial availability of anthocyanidin standards, naturally occurring anthocyanins have been used as the main source in most studies to identify anthocyanidins *in vitro*. In practice, authentic anthocyanidins are needed for all anthocyanin-related studies. A novel approach to the stabilization of anthocyanidins involves their deposition in C₁₈ SPE cartridges. Dao et al. (1998) have shown that the amount of anthocyanidins, delphinidin, petunidin, and malvidin was unchanged for the first 7 days of storage in SPE cartridges under nitrogen atmosphere at 2°C. After 45 days of storage the levels of delphinidin, petunidin, and malvidin were reduced to 82, 63, and 49% of the original content, respectively. In contrast, the level of delphinidin, petunidin, and malvidin decreased to 17, 10, and 3%, respectively, of the original amount when these anthocyanidins were stored for 3 days in acidic methanol (0.01% HCl) at 2°C.

5.3.5 COUNTERCURRENT CHROMATOGRAPHY

Countercurrent chromatography (CCC) is a support-free, all-liquid chromatographic technique that relies on the partition of a sample between two immiscible phases. This technique has the following advantages over other chromatographic techniques: (1) no irreversible adsorption of the sample (since no solid support is employed), (2) quantitative recovery of the sample, and (3) sample capacity is high due to the large volumes of stationary phase used (up to several hundred milligrams of pure compounds can be achieved in a single CCC run).

Droplet countercurrent chromatography (DCCC) with acid-containing solvents has been employed for the separation of anthocyanins. Francis and Andersen (1984) tested the suitability of three solvent systems: CMH, chloroform–methanol–0.1 *N* hydrochloric acid (5:6:4); PAH, *n*-propanol–acetic acid–0.1 *N* hydrochloric acid (3:4:3); and BAW, butanol–acetic acid–water (4:1:5) for the separation of anthocyanins by DCCC. The BAW system was found to be the most effective in separating anthocyanins from black currants (*Ribes nigrum* L.) and raspberries (*Rubus idaeus* L.). They suggested that the upper phase of this solvent system (BAW) be used for the separation of less polar anthocyanins, whereas the lower phase be employed for the separation of more polar anthocyanins. Andersen and co-workers (1991) used

the following procedure to isolate cyanidin 3-sambubioside from the fruits of the European elderberry *Sambucus nigra* (Caprifoliaceae). Fruits were extracted with acidified methanol and then partitioned against ethyl acetate. The polar fraction was chromatographed on an Amberlite XAD-7 column and then injected onto an Eyela Tokyo Rikakikai Model A DCCC instrument (containing 300 glass columns, 40 cm $\times$ 2 mm). Elution was achieved with the lower phase of *n*-butanol-acetic acid-water (4:1:5). Final purification of the anthocyanin was performed by another Amberlite XAD-7 step followed by Sephadex LH-20 column chromatography.

Centrifugal partition chromatography (CPC), a type of CCC that uses discrete partition cells inside a rotor, was used by Renault et al. (1997) to separate anthocyanins from black currant and grape skins. They used two instruments from Sanki Engineering Ltd. (Kyoto, Japan), a laboratory model LLB-M and a pilot scale model LLI-7, both fitted with a stacked disk-type rotor.

The separation of anthocyanins using high-speed countercurrent chromatography (HSCCC) was impressively demonstrated by Degenhardt, Knapp, and Winterhalter (2000). A high-speed model CCC-1000 (Pharma-Tech Research Corp., Baltimore, MD), equipped with three preparative coils connected in series (total volume = 850 ml, tubing diameter = 2.6 mm), was employed. These researchers used a biphasic mixture of *tert*-butyl methyl ether-*n*-butanol-acetonitrile-water (2:2:1:5) acidified with TFA as the solvent system and a revolution speed of 1000 rpm to effectively fractionate anthocyanins mixtures from red cabbage, black currant, black chokeberry, and roselle. Figure 5.4 shows the HSCCC separation of black currant anthocyanins. The elution mode was head to tail with the upper layer being the stationary phase. They were able to separate anthocyanins from extracts without any sample pretreatment. However, minor impurities were observed in the fractionated anthocyanins [by nuclear magnetic resonance (NMR)] so the authors recommended cleanup of the extracts by XAD-7 column chromatography prior to separation by HSCCC.

5.3.6 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC has revolutionized anthocyanin studies due to its high efficiency, sensitivity, speed, and accurate quantitation (Andersen, 1985, 1987a,b; Baj et al., 1983; Castelee et al., 1983; Hong and Wrolstad, 1986, 1990a,b; Strack and Wray, 1989; Wilkinson, Swenny, and Iacobucci, 1977; Wulf and Nagel, 1978). Preparative HPLC has been shown to be a rapid and gentle means of purifying anthocyanins from complex mixtures, while analytical HPLC is useful in determining the purity of isolated fractions and in determining the quantitative distribution of anthocyanins in natural extracts. Applications are typically performed on C₁₈ (silica-based) columns using acidic solvents such as acetic, formic, or phosphoric acid in water-methanol or water-acetonitrile mixtures as the mobile phase. Polymeric reversed-phase columns (polystyrene-divinylbenzene; PLRP-S column, Polymer Laboratories Inc., Amherst, MA), used by Wrolstad and co-workers (Hong and Wrolstad, 1990b; Rodriguez-Sanona, Giusti, and Wrolstad, 1998) have the advantage of low pH stability. This permits the use of phosphoric acid-water and acetonitrile mobile phases which have a lower UV cutoff than acetic acid-water-methanol mixtures. This may be less of a problem for reversed-phase columns available today which feature improved pH

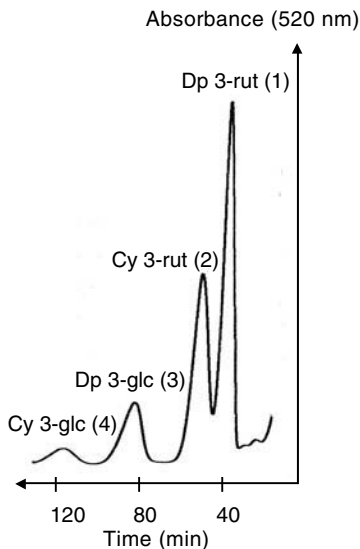


FIGURE 5.4 HSCCC separation of black currant (*Ribes nigrum* L.) anthocyanins on a Pharma-Tech Research Corp. model CCC-1000 (three coils, total volume = 850 ml). Solvent system: *tert*-butyl methyl ether–*n*-butanol–acetonitrile–water (2:2:1:5) acidified with TFA; speed: 1000 rpm; elution mode: head to tail; stationary phase: upper layer; peak identities: (1) delphinidin 3-rutinoside, (2) cyanidin 3-rutinoside, (3) delphinidin 3-glucoside, (4) cyanidin 3-glucoside. (Reprinted with permission from Degenhardt, A., Knapp, H., and Winterhalter, P., *J. Agric. Food Chem.*, 48, 338–343, 2000. Copyright 2000 American Chemical Society.)

stability due to three innovations: (1) introduction of bulky hydrophobic groups on the silane (to hinder access of the mobile phase to the siloxane bond), (2) use of bonded phases with multiple attachment points to the silica surface (making hydrolysis at a given anchoring site less likely to cause phase loss), and (3) use of hybrid organic-inorganic particles (alkyl groups are incorporated into the silica matrix improving both low and high pH stability) (Wehr, 2000). Various HPLC and sample preparation methods for the measurement of food flavonoids, including anthocyanins and anthocyanidins, have been tabulated in a recent review of Merken and Beecher (2000). Structural effects on HPLC retention (C_{18} silica-based columns) can be summarized as follows: hydroxylation and glycosylation result in shorter retention times, while methylation and acylation cause longer retention times. The HPLC separation of black bean anthocyanins is shown in Figure 5.5.

5.4 METHODS OF IDENTIFICATION

5.4.1 HYDROLYSIS

The first step in anthocyanin characterization is the identification of the anthocyanidin moiety (aglycon) and the sugar(s). This can be accomplished by acid hydrolysis followed by chromatography of the products with reference standards. Hydrolysis of anthocyanins can be accomplished by heating the sample with 2 *N* HCl in methanol (1:1) under reflux for 60 min and then cooling in an ice bath. The anthocyanidins

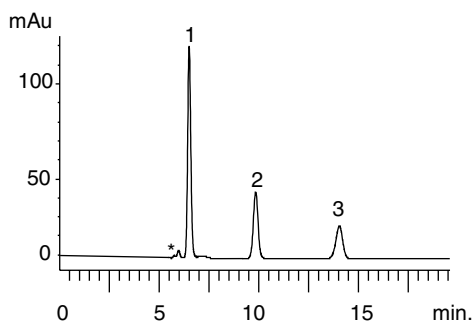


FIGURE 5.5 HPLC chromatogram of black bean anthocyanins monitored at 520 nm. Peak identities: (1) dephinidin 3-glucoside, (2) petunidin 3-glucoside, (3) mavidin 3-glucoside, (*) unknown anthocyanin; column: Keystone ODS/B 25 $\times$ 4.6 mm ID; solvent A: 10% formic acid; solvent B: formic acid–methanol–water (10:40:50, v/v); linear gradient from 40 to 80% solvent B in 50 min.

can then be extracted with ethyl acetate, evaporated to dryness under a stream of nitrogen, redissolved in 10% formic acid, filtered through a 0.5- μ m disposable membrane filter, and promptly analyzed by HPLC (Takeoka et al., 1997).

5.4.2 CHROMATOGRAPHY

TLC, HPLC, and capillary gas chromatography (GC) are effective methods of identifying carbohydrates liberated by the acid hydrolysis of anthocyanins. Carbohydrate samples must be derivatized prior to analysis by capillary GC and GC-mass spectrometry (MS). We have employed methyloxime-trimethylsilyl (MO-TMS) derivatives of carbohydrates for these analyses (Takeoka et al., 1997) using the following procedure. A mixture of dry pyridine (125 μ l) and 1 mg of *O*-methylhydroxylamine-HCl was added to 1 mg of each carbohydrate sample and standard. The mixtures were mixed well, heated for 2 h at 40°C, and allowed to stand overnight at room temperature. Pyridine was removed in a stream of nitrogen while the sample was heated to 40°C. When the solution approached dryness, two drops of benzene were added to azeotropically remove the last traces of water formed in the oximation reaction. One hundred microliters of a 99:1 mixture of *N,O*-bis(trimethylsilyl)tri-fluoroacetamide (BSTFA) plus trimethylchlorosilane (TMCS) (Sylon BFT, Supelco, Inc., Bellefonte, PA) was added to each sample. The mixtures were shaken well, heated at 40°C for 2 h, and allowed to stand overnight at room temperature. This procedure is a modification of the method of Laine and Sweeley (1971, 1973) for the preparation of MO-TMS derivatives of aldoses, partially methylated aldoses, deoxyaldoses, and ketoses. Samples can be subsequently analyzed by capillary GC using a nonpolar stationary phase such as DB-1 (J&W Scientific, Folsom, CA). Polar stationary phases such as DB-Wax are not recommended due to possible reaction of the silylating agent with the stationary phase.

Hong and Wrolstad (1990a) have effectively used a combination of retention properties on reversed-phase HPLC and spectral properties to characterize anthocyanins in various colorants and fruit juices.

5.4.3 SPECTROSCOPY

5.4.3.1 UV-Visible Adsorption Spectroscopy

The spectral characteristics of anthocyanins give important information with regard to the nature of the aglycon and the type of glycosylation. The spectra of anthocyanins are typically measured in methanol containing 0.01% HCl. The type of aglycon present can be inferred from the visible $\lambda_{\max}$. Pelargonidin 3-glucosides have a visible $\lambda_{\max}$ at about 505 nm; cyanidin and peonidin 3-glucosides exhibit their $\lambda_{\max}$ at 520 to 526 nm, while all delphinidin derivatives (delphinidin, malvidin, petunidin) display their $\lambda_{\max}$ at 532 to 537 nm (Harborne, 1967). Anthocyanidins typically display a 6- to 12-nm larger visible $\lambda_{\max}$ in 0.01% methanolic HCl than the corresponding anthocyanins (Harborne, 1958). Pelargonidin derivatives have a visible $\lambda_{\max}$ at 512 nm; cyanidin and peonidin derivatives exhibit their $\lambda_{\max}$ at 523 to 525 nm, while delphinidin derivatives display their $\lambda_{\max}$ at 529 to 533 nm (Hong and Wrolstad, 1990b). When measuring the spectrum on-line (i.e., diode array detector), as the anthocyanin elutes from the HPLC column the solvent composition is limited by the mobile phase composition. Since the spectral characteristics of the anthocyanins are dependent on both the pH and the nature of the solvent, direct comparison of spectral characteristics in different solvent systems may not be appropriate. In general, there is a 15-nm shift toward shorter wavelengths when water is substituted for methanol (Harborne, 1958). Using HPLC combined with diode array detection, Hong and Wrolstad (1990a) have shown that acylated (with hydroxy aromatic acids) cyanidin glycosides tend to have a much higher wavelength maximum than their nonacylated counterparts. They attributed this difference (as much as 20 nm in some cases) to the predominantly aqueous HPLC solvent system, since earlier studies with anthocyanins in acidified methanol showed only small differences in the visible wavelength maximum between acylated and nonacylated anthocyanins with the same aglycon (Hrazdina, Iredale, and Mattick, 1977; Wulf and Nagel, 1978). The glycosidic substitution pattern of anthocyanins can be elucidated by absorption in the 400- to 460-nm region since 3,5- and 5-glycosides exhibit ratios of $A_{440}/A_{\lambda_{\max}}$ about 50% of those of 3-glycosides and the free anthocyanidins (Harborne, 1967). Increasing the solvent polarity changes the spectral characteristics of anthocyanins by increasing the $A_{440}/A_{\lambda_{\max}}$ ratio and decreasing the visible $\lambda_{\max}$ (Andersen, 1985, 1987a; Hong and Wrolstad, 1990b). It has been reported that compounds possessing a disaccharide as a substituent group in the 3-position of the chromophore exhibit a large drop in absorptivity (Figueiredo et al., 1996a,b; Elhabiri et al., 1995). These workers found that cyanidin 3-sambubioside-5-glucoside, delphinidin 3-gentiobioside, and cyanidin 3-rutinoside showed a marked hypsochromic effect when compared to the corresponding 3-glucosides and 3,5-diglucosides with a similar substitution pattern. In their study of pelargonidin derivatives, Giusti et al. (1999b) reported a small decrease in the molar absorptivity when a glucose unit was attached to the 3-position as compared to the aglycon. Addition of other glucose units produced a hyperchromic effect as pelargonidin 3-sophoroside-5-glucoside had a substantially higher molar absorptivity than pelargonidin 3-glucoside (Table 5.3). The presence of acylation with hydroxylated aromatic acids can be deduced by the appearance of a peak in

TABLE 5.3
Absorptivity Coefficients ($\text{l cm}^{-1} \text{ mol}^{-1}$) of Different Pelargonidin Derivatives^a

Anthocyanin ^b	λ_{max}		Molar Absorptivity	
	MeOH ^c	Buffer ^d	MeOH ^a	Buffer ^c
Pg aglycon	524	505	19,780	18,420
Pg 3-glu	508	496	17,330	15,600
Pg 3-soph-5-glu	506	497	30,700	25,370
Pg 3-soph-5-glu + p-coumaric	508	506	34,890	28,720
Pg 3-soph-5-glu + ferulic	507	506	29,640	24,140
Pg 3-soph-5-glu + p-coumaric and malonic	508	508	39,780	33,020
Pg 3-soph-5-glu + ferulic and malonic	508	508	39,380	31,090
Pg 3-rut-5-glu + p-coumaric	511	504	39,590	32,080

^a Pg, pelargonidin; glu, glucose; soph, sophoroside.

^b Adapted from Giusti et al. (1999b).

^c 0.1% HCl in methanol.

^d 0.2 N KCl, pH 1.0.

the 310- to 335-nm range. The position of the acyl λ_{max} gives an indication of the nature of the acylating acid, while the $A_{\lambda_{\text{max}}}(\text{acyl})/A_{\lambda_{\text{max}}}(\text{visible})$ ratio is a measure of the number of aromatic acids present in the anthocyanin (Harborne, 1967). Harborne (1958) noted that in acidified methanolic solutions, an $A_{\lambda_{\text{max}}}(\text{acyl})/A_{\lambda_{\text{max}}}(\text{visible})$ ratio of 48 to 71% is characteristic of a 1:1 molar ratio of cinnamic acid to anthocyanin, while an $A_{\lambda_{\text{max}}}(\text{acyl})/A_{\lambda_{\text{max}}}(\text{visible})$ ratio of 83 to 107% is indicative of a 2:1 molar ratio of cinnamic acid to anthocyanin.

5.4.3.2 Mass Spectrometry

Fast atom bombardment (FAB)-MS became a useful technique for anthocyanin structural studies in the 1980s (Harborne and Grayer, 1988; Strack and Wray, 1989). The sample is dissolved in a nonvolatile matrix such as glycerol or a mixture of thioglycerol–diglycerol (1:1) and placed on a direct insertion probe. The matrix facilitates desorption as well as ionization. FAB desorption is accomplished by bombarding the sample solution with a high-energy beam of xenon atoms or cesium ions, causing sample desorption (often as an ion) by momentum transfer. Saito et al. (1983) were the first group to perform FAB-MS on anthocyanins. This technique has been widely used for molecular weight determination of anthocyanins (Bakker, Spomer, and Berber-Jiménez, 1997; Bakker and Timberlake, 1997; Baublis et al., 1994; Fossen et al., 1996; Kim et al., 1989; Saito et al., 1995; Takeda, Harborne, and Self, 1986). However, FAB has not been as successful for the analysis of anthocyanidins. In contrast, plasma desorption mass spectrometry (PDMS) has been shown to be effective in the analysis of anthocyanidins that are hydroxylated at the 3-position (Wood et al., 1993). In PDMS the sample is deposited on a thin metal foil, typically nickel. Spontaneous fission of a radioactive ^{252}Cf source produces fission fragments such as $^{142}\text{Ba}^{18+}$ and $^{106}\text{Tc}^{22+}$ which have kinetic energies of roughly

79 and 104 MeV, respectively (Williams and Fleming, 1995). Passage of a high-energy fission fragment through the sample foil results in extremely rapid localized heating that desorbs the sample molecules and generates ions. These ions are then accelerated out of the source and into the mass analyzer. PDMS has also been successfully used to identify and characterize both anthocyanins and 3-deoxyanthocyanidins, though it has been found that acetic acid interferes with the analysis (Wood et al., 1994). With regard to sensitivity, it has been shown that 1-ng levels of pelargonidin chloride could be detected with an observed ion, m/z 271 (corresponding to the flavylum cation), using PDMS (Wood et al., 1993). It was necessary in most cases to partially purify the samples by HPLC or by partitioning with organic solvents prior to PDMS analysis.

Matrix-assisted laser desorption/ionization (MALDI) coupled with time-of-flight (TOF) mass analysis has emerged as a particularly useful tool for the analysis of biomolecules (Cotter, 1992). In this method, the sample is first cocrystallized with a large molar excess of a matrix compound that is selected to strongly absorb laser light. Typical matrix compounds are 2,5-dihydroxybenzoic acid and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid). Pulse UV laser radiation of the sample-matrix mixture results in vaporization of the matrix which carries the sample with it. Sample degradation is avoided since energy transfer is indirect and in a controlled manner. MALDI-TOF has had its greatest impact in the area of protein research, since it can give approximate molecular weight determinations up to 100,000 to 200,000 Da (Williams and Fleming, 1995). MALDI-TOF has been employed in the analysis of mixtures of 3-deoxyanthocyanins and anthocyanins (Sugui et al., 1998). For sample preparation, these workers solubilized the matrix α -cyano-4-hydroxycinnamic acid (10 mg) in 1 ml of a mixture of 1% TFA-H₂O-CH₃CN (1:4:5). The samples were mixed with the matrix for 1 min and then the resulting mixture was applied to the sample plate. The solvents were allowed to evaporate at room temperature. Sensitivities of 15 pmol μl^{-1} were achieved for 3-deoxyanthocyanidins in extracts. MALDI-MS with a Proflex III linear mode (Bruker Analytical Systems, Inc., Billerica, MA) was used to analyze anthocyanins in red wine and fruit juice (Wang and Sporns, 1999). These researchers used 2,4,6-trihydroxyacetophenone as the matrix. It was shown that monoglucoside anthocyanins had a similar response, while a diglucoside anthocyanin, e.g., malvidin 3,5-diglucoside, or an anthocyanin with a disaccharide attached to the 3-position, e.g., cyanidin 3-rutinoside, had relative molar responses only one fourth that of the monoglucosylated anthocyanins (Wang and Sporns, 1999). Anthocyanins were extracted from wine and fruit juice samples using Sep-pak C₁₈ cartridges prior to MALDI-MS analysis.

Electrospray MS may be the most promising ionization technique for the characterization of anthocyanins (Giusti et al., 1999a). Electrospray ionization is the most useful and versatile ionization technique in MS (Covey, 1995). The only absolute requirement for ionization is that the constituents of interest must be soluble in some solvent. Electrospray ionization generates ions directly from solution by creating a spray or fine mist of highly charged droplets in the presence of a strong electrical field (3 to 6 kV). If the spray contains sample molecules, a molecular ion of the sample molecules can be generated by evaporation of the solvent. This is

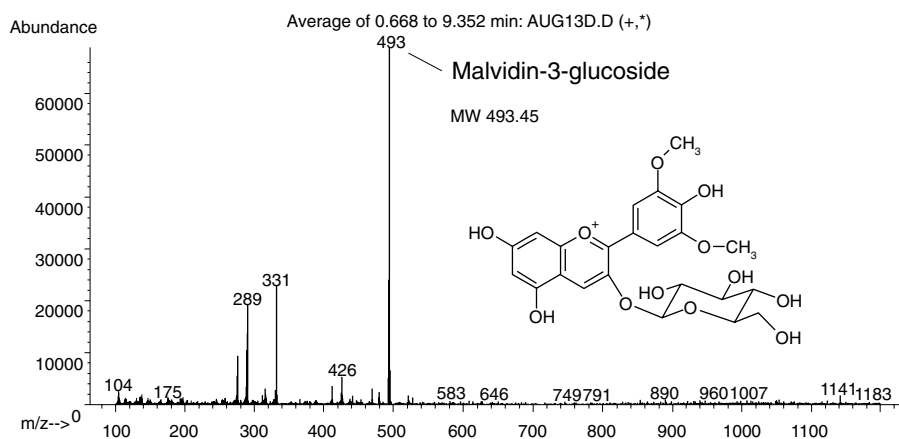


FIGURE 5.6 Electrospray mass spectrum of malvidin 3-glucoside with m/z 493 representing the molecular cation and m/z 331 representing the malvidin cation. The sample was run on an Extrel C-50/LQ-400 hybrid, single-quadrupole mass spectrometer equipped with an electrospray source from Analytica of Bradford, Inc. (Bradford, PA). The sample was diluted in a solution of water–acetonitrile–acetic acid (40:10:50, v/v/v) and introduced into the instrument at a flow rate of 1.3 $\mu\text{L}/\text{min}$ via a syringe pump (Harvard Apparatus, Model 11, South-natick, MA).

achieved by passing a drying gas across the spray. In the process of evaporation droplets are reduced in size, while the electric charge density on their surface increases. Desolvation may then be aided by repulsive Coulombic forces overcoming the cohesive forces of the droplet. Sample molecules eventually leave the liquid phase and become gas-phase ions in the atmospheric region of the ion source. The ions are then electrostatically directed into the mass analyzer. The positive charge of anthocyanins at low pH values allows for their easy detection at low voltages since other interfering compounds are usually not ionized (Giusti et al., 1999a). Figure 5.6 shows an electrospray mass spectrum of mavidin 3-glucoside. The ions displayed at m/z 493 [corresponding to the mass calculated for $\text{C}_{23}\text{H}_{25}\text{O}_{12}$ (m/z 493.448)] and 331 represent the molecular cation M^+ and the malvidin cation, respectively. Alternatively, samples can be introduced with minimal clean up (C_{18} SPE) directly into the mass spectrometer allowing the effective molecular weight determination of anthocyanins present in extracts. However, an intermediate step of washing the C_{18} SPE cartridge with ethyl acetate (to remove interfering phenolic acids and flavonoids) prior to elution with acidified methanol was found to produce much cleaner MS profiles and was recommended (Giusti et al., 1999a). Liquid chromatography-mass spectrometry (LC-MS) may be the ideal technique for the rapid characterization of anthocyanins (Miller et al., 1998). Standard formic acid–water–methanol gradients are readily adapted to LC-MS with formic acid facilitating ionization. The natural molecular cations M^+ are readily observed in the positive ion mode. In source collision-induced dissociation (CID) can be used to fragment the anthocyanin ion to elucidate the aglycon. Alternatively, electrospray ionization with an ion trap (Degenhardt, Knapp, and Winterhalter, 2000; Piovani,

Filippini, and Favretto, 1998) or a triple-stage quadrupole (Giusti et al., 1999a) may be employed to elucidate characteristic fragments of the molecular ion. Gläbgen et al. (1992a) applied NMR and pneumatically assisted electrospray (ion spray) to characterize six anthocyanins from cell cultures of an Afghan cultivar of *Daucus carota*. They used a Sciex API III triple quadrupole mass spectrometer equipped with an ion spray ion source (PE Sciex, Foster City, CA). Extracts and purified anthocyanins were dissolved in MeOH–HOAc–H₂O (50:8:42) and introduced directly into the ion spray source at a constant flow rate of 5 µl/min with a medical infusion syringe pump (Harvard Apparatus, Southnatick, MA). The system could detect 4.49 ng (10 pmol) of cyanidin 3-galactoside (molecular ion) with a signal-to-noise ratio of 3:1 under full scan conditions (m/z 100 to 1200 at a scan rate of 5 sec/scan) (Gläbgen, Seitz, and Metzger, 1992b).

5.4.3.3 Nuclear Magnetic Resonance Spectroscopy

Modern one- and two-dimensional NMR spectroscopy represents the most powerful tool in the structural elucidation of complex anthocyanins. Andersen and co-workers (1991) were the first to apply a heteronuclear shift correlation experiment to the structural characterization of an anthocyanin. Giusti, Ghanadan, and Wrolstad (1998) recently used one- and two-dimensional NMR techniques for structural conformation of two diacylated anthocyanins, pelargonidin 3-*O*-[2-*O*-(β-glucopyranosyl)-6-*O*-(*trans-p*-coumaroyl)-β-glucopyranoside]5-*O*-(6-*O*-malonyl-β-glucopyranoside) and pelargonidin 3-*O*-[2-*O*-(β-glucopyranosyl)-6-*O*-(*trans-feruloyl*)-β-glucopyranoside]5-*O*-(6-*O*-malonyl-β-glucopyranoside isolated from red radish (*Raphanus sativus*). Nuclear Overhauser effect spectroscopy (NOESY) revealed spatial proximity between hydrogens from the cinnamic acid acylating group and the C-4 of the pelargonidin. These results suggest folding of the acylated anthocyanins which may be an important factor in their increased color stability.

Detailed ¹H NMR spectroscopy of flavonoids and their glycosides (including anthocyanins) was reviewed in a chapter by Markham and Geiger (1994). Other useful information on NMR of anthocyanins can be found in Strack and Wray (1989, 1994).

REFERENCES

- Andersen, Ø.M. Chromatographic separation of anthocyanins in Crowberry (Lignonberry) *Vaccinium vitis-Idaea* L., *J. Food Sci.*, 50, 1230–1232, 1985.
- Andersen, Ø.M. Anthocyanins in fruits of *Vaccinium uliginosum* L. (bog whortleberry), *J. Food Sci.*, 52, 665–667, 1987a.
- Andersen, Ø.M. Anthocyanins in fruits of *Vaccinium japonicum*, *Phytochemistry*, 26, 1220–1221, 1987b.
- Andersen, Ø.M. Semipreparative isolation and structure determination of pelargonidin 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside and other anthocyanins from the tree *Dacrycarpus dacrydiodes*, *Acta Chem. Scand.*, B42, 462–468, 1988.
- Andersen, Ø.M., Aksnes, D.W., Nerdal, W., and Johansen, O.P., Structural elucidation of cyanidin-3-sambubioside and assignments of the ¹H and ¹³C NMR resonances through two-dimensional shift-correlated NMR techniques, *Phytochem. Anal.*, 2, 175–183, 1991.

- Andersen, Ø.M. and Francis, G.W., Simultaneous analysis of anthocyanins and anthocyanidins on cellulose thin layers, *J. Chromatogr.*, 318, 450–454, 1985.
- Asen, S., Preparative thin-layer chromatography of anthocyanins, *J. Chromatogr.*, 18, 602–603, 1965.
- Baj, A., Bombardelli, E., Gabetta, B., and Martinelli, E.M., Qualitative and quantitative evaluation of *Vaccinium myrtillus* anthocyanins by high-resolution gas chromatography, *J. Chromatogr.*, 279, 365–372, 1983.
- Bakker, J., Bridle, P., Honda, T., Kuwano, H., Saito, N., Terahara, N., and Timberlake, C.F., Identification of an anthocyanin occurring in some red wines, *Phytochemistry*, 44, 1375–1382, 1997.
- Bakker, J. and Timberlake, C.F., The distribution of anthocyanins in grape skin extracts of port wine cultivars as determined by high performance liquid chromatography, *J. Sci. Food Agric.*, 36, 1315–1324, 1985.
- Bakker, J. and Timberlake, C.F., Isolation, identification, and characterization of new color-stable anthocyanins occurring in some red wines, *J. Agric. Food Chem.*, 45, 35–43, 1997.
- Bate-Smith, E.C., Leuco-anthocyanins. 1. Detection and identification of anthocyanins formed from leuco-anthocyanins in plant tissues, *Biochem. J.*, 58, 122–125, 1954.
- Baublis, A., Spomer, A., and Berber-Jiménez, M.D. Anthocyanin pigments: comparison of extract stability, *J. Food Sci.*, 59, 1219–1221, 1994.
- Bohm, B.A., *Introduction to Flavonoids*, Harwood Academic Publishers, Amsterdam, 1998.
- Brouillard, R., Flavonoids and flower color, in *The Flavonoids: Advances in research since 1980*, Harborne, J.B., Ed., Chapman & Hall, London, 1988, pp. 525–538.
- Cabrita, L. and Andersen, Ø.M., Anthocyanins in blue berries of *Vaccinium padifolium*. *Phytochemistry*, 52, 1693–1696, 1999.
- Castele, K.V., Gieger, H., De Loose, R., and Van Sumere, C.F., Separation of some anthocyanidins, anthocyanins, proanthocyanidins and related substances by reversed-phase high-performance liquid chromatography, *J. Chromatogr.*, 259, 291–300, 1983.
- Catalano, G., Fossen, T., and Andersen, Ø.M., Petunidin 3-*O*- α -rhamnopyranoside-5-*O*- β -glucopyranoside and other anthocyanins from flowers of *Vicia villosa*, *J. Agric. Food Chem.*, 46, 4568–4570, 1998.
- Cotter, R.J., Time-of-flight mass spectrometry for the structural analysis of biological molecules, *Anal. Chem.*, 64, 1027A–1039A, 1992.
- Covey, T., Analytical characteristics of the electrospray ionization process, in *Biochemical and Biotechnological Applications of Electrospray Ionization Mass Spectrometry*, Snyder, A.P., Ed., ACS Symposium Series 619, American Chemical Society, Washington, D.C., 1995, pp. 21–59.
- Dao, L.T., Takeoka, G.R., Edwards, R.H., and Berrios, J. de J., An improved method for the stabilization of anthocyanidins, *J. Agric. Food Chem.*, 46, 3564–3569, 1998.
- Degenhardt, A., Knapp, H., and Winterhalter, P., Separation and purification of anthocyanins by high-speed countercurrent chromatography and screening for antioxidant activity, *J. Agric. Food Chem.*, 48, 338–343, 2000.
- Elhabiri, M., Figueiredo, P., Fougereuse, A., and Brouillard, R., A convenient method for conversion of flavonoids into anthocyanins, *Tetrahedron Lett.*, 36, 4611–4614, 1995.
- Figueiredo, P., Elhabiri, M., Saito, N., and Brouillard, R., Anthocyanin intramolecular interactions. A new mathematical approach to account for the remarkable colorant properties of the pigments extracted from *Matthiola incana*, *J. Am. Chem. Soc.*, 118, 4788–4793, 1996a.
- Figueiredo, P., Elhabiri, M., Toki, K., Saito, N., Dangles, O., and Brouillard, R., New aspects of anthocyanin complexation. Intramolecular copigmentation as a means for colour loss? *Phytochemistry*, 41, 301–308, 1996b.

- Fossen, T. and Andersen, Ø.M., Acylated anthocyanins from leaves of the water lily, *Nymphaeæ X marliacea*, *Phytochemistry*, 46, 353–357, 1997a.
- Fossen, T. and Andersen, Ø.M., Malonated anthocyanins of garlic *Allium sativum* L., *Food Chem.*, 58, 215–217, 1997b.
- Fossen, T., Andersen, Ø.M., Øvstedal, D.O., Pedersen, A.T., and Raknes, Å., Characteristic anthocyanin pattern from onions and other *Allium* spp., *J. Food Sci.*, 61, 703–706, 1996.
- Francis, G.W. and Andersen, Ø.M., Droplet counter-current chromatography of anthocyanins, *J. Chromatogr.*, 283, 445–448, 1984.
- Giusti, M.M., Ghanadan, H., and Wrolstad, R.E., Elucidation of the structure and conformation of red radish (*Raphanus sativus*) anthocyanins using one- and two-dimensional nuclear magnetic resonance techniques, *J. Agric. Food Chem.*, 46, 4858–4863, 1998.
- Giusti, M.M., Rodríguez-Saona, L.E., Griffin, D., and Wrolstad, R.E., Electrospray and tandem mass-spectroscopy as tools for anthocyanin characterization, *J. Agric. Food Chem.*, 47, 4657–4664, 1999a.
- Giusti, M.M., Rodríguez-Saona, L.E., and Wrolstad, R.E., Molar absorptivity and color characteristics of acylated and non-acylated pelargonidin-based anthocyanins, *J. Agric. Food Chem.*, 47, 4631–4637, 1999b.
- Giusti, M.M. and Wrolstad, R.E., Characterization of red radish anthocyanins, *J. Food Sci.*, 61, 322–326, 1996.
- Gläbgen, W.E., Wray, V., Strack, D., Metzger, J.W., and Seitz, H.U., Anthocyanins from cell suspensions of *Daucus carota*, *Phytochemistry*, 31, 1593–1601, 1992a.
- Gläbgen, W.E., Seitz, H.U., and Metzger, J.W., High-performance liquid chromatography/electrospray mass spectrometry and tandem mass spectrometry of anthocyanins from plant tissues and cell cultures of *Daucus carota* L., *Biol. Mass Spectrom.*, 21, 271–277, 1992b.
- Harborne, J.B., Spectral methods of characterizing anthocyanins, *Biochem. J.*, 70, 22–28, 1958.
- Harborne, J.B., *Comparative Biochemistry of the Flavonoids*. Academic Press, London, 1967.
- Harborne, J.B., Ed., *The Flavonoids: Advances in Research Since 1980*. Chapman & Hall, London, 1988.
- Harborne, J.B., Ed., *The Flavonoids: Advances in Research Since 1986*, Chapman & Hall, London, 1994.
- Harborne, J.B., *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis*, 3rd ed., Chapman & Hall, London, 1998.
- Harborne, J.B. and Boardly, M., The widespread occurrence in nature of anthocyanins as zwitterions, *Z. Naturforsch.*, 40c, 305, 1985.
- Harborne, J.B. and Mabry, T.J., Eds., *The Flavonoids: Advances in Research*, Chapman & Hall, London, 1982.
- Harborne, J.B. and Grayer, R., *The Flavonoids: Advances in Research Since 1980*, Harborne, J.B., Ed., Chapman & Hall, New York, pp. 1–20, 1988.
- Harborne, J.B., Mabry, T.J., and Mabry, H., Eds., *The Flavonoids*, Part 1, Academic Press, New York, 1975.
- Henke, H., *Preparative Gel Chromatography on Sephadex LH-20*. Hüthig, Heidelberg, 1995.
- Hong, V. and Wrolstad, R.E., Cranberry juice composition, *J. Assoc. Off. Anal. Chem.*, 69, 199–207, 1986.
- Hong, V. and Wrolstad, R.E., Characterization of anthocyanin-containing colorants and fruit juices by HPLC/photodiode array detection, *J. Agric. Food Chem.*, 38, 698–708, 1990a.
- Hong, V. and Wrolstad, R.E., Use of HPLC/photodiode array detection for the characterization of anthocyanins, *J. Agric. Food Chem.*, 38, 708–715, 1990b.

- Hrazdina, G., Iredale, H., and Mattick, L.R., Anthocyanin composition of *Brassica oleracea* cv. Red Danish, *Phytochemistry*, 16, 297–299, 1977.
- Iacobucci, G.A. and Sweeney, J.G., The chemistry of anthocyanins, anthocyanidins and related flavylum salts, *Tetrahedron*, 39, 3005–3038, 1983.
- Jackman, R., Yada, R.Y., and Tung, M.A., A review: separation and chemical properties of anthocyanins used for their qualitative and quantitative analysis, *J. Food Biochem.*, 11, 279, 1987.
- Kidøy, L., Nygård, A.M., Andersen, Ø.M., Pedersen, A.T., Aksnes, D.W., and Kiremire, B.T., Anthocyanins in fruits of *Passiflora edulis* and *P. suberosa*, *J. Food Comp. Anal.*, 10, 49–54, 1997.
- Kim, J.H., Nonaka, G.-I., Fujieda, K., and Uemoto, S., Anthocyanin malonylglucosides in flowers of *Hibiscus syriacus*, *Phytochemistry*, 28, 1503–1506, 1989.
- Kondo, T., Nakane, Y., Tamura, H., Goto, T., and Eugster, C.H., Structure of monardaein, a bis-malonylated anthocyanin isolated from golden balm, *Monarda didyma*, *Tetrahedron Lett.*, 26, 5879–5882, 1985.
- Kraemer-Schafhalter, A., Fuchs, H., and Pfannhauser, W., Solid-phase extraction (SPE) — a comparison of 16 materials for the purification of anthocyanins from *Aronia melanocarpa* var Nero, *J. Sci. Food Agric.*, 78, 435–440, 1998.
- Laine, R.A. and Sweeley, C.C., Analysis of trimethylsilyl *O*-methyloximes of carbohydrates by combined gas-liquid chromatography–mass spectrometry, *Anal. Biochem.*, 43, 533–538, 1971.
- Laine, R.A. and Sweeley, C.C., *O* Methyloximes of sugars. Analysis as *O*-trimethylsilyl derivatives by gas-liquid chromatography and mass spectrometry, *Carbohydrate Res.*, 27, 199–213, 1973.
- Main, J.H., Clydesdale, F.M., and Francis, F.J., Spray drying anthocyanin concentrates for use as food colorants, *J. Food Sci.*, 43, 1693–1694, 1697, 1978.
- Markham, K.R., *Techniques of Flavonoid Identification*. Academic Press, New York, 1982.
- Markham, K.R., Novel anthocyanins produced in petals of genetically transformed lisianthus, *Phytochemistry*, 42, 1035–1038, 1996.
- Markham, K.R. and Geiger, H., ¹H nuclear magnetic resonance spectroscopy of flavonoids and their glycosides in hexadeuterodimethylsulfoxide, in *The Flavonoids: Advances in Research Since 1986*, Harborne, J.B., Ed., Chapman & Hall, London, pp. 441–497, 1994.
- Mazza, G. and Miniati, E., *Anthocyanins in Fruits, Vegetables, and Grains*, CRC Press, Boca Raton, 1993.
- Merken, H.M. and Beecher, G.R., Measurement of food flavonoids by high-performance liquid chromatography: a review, *J. Agric. Food Chem.*, 48, 577–599, 2000.
- Miller, C.A., Fischer, S.M., Kalt, W., McDonald, J.E., and Ricker, R.D., LC/MS analysis of anthocyanins. Presented at ASMS '98, Orlando, FL, 1998.
- Nørbæk, R. and Kondo, T., Anthocyanins from flowers of crocus (Iridaceae), *Phytochemistry*, 47, 861–864, 1998.
- Ohta, H., Akuta, S., and Osajima, Y., Stability of anthocyanin pigments and related compounds in acidic solutions, *Nippon Shokuhin Kogyo Gakkai Shi*, 27, 81–85, 1980.
- Oszmianski, J. and Lee, C.Y., Isolation and HPLC determination of phenolic compounds in red grapes, *Am. J. Enol. Vitic.*, 41(3), 204–206, 1990.
- Philip, T., An anthocyanin recovery system from grape wastes, *J. Food Sci.*, 39, 859, 1974.
- Piovan, A., Filippini, R., and Favretto, D., Characterization of the anthocyanins of *Catharanthus roseus* (L.) G. Don *in vivo* and *in vitro* by electrospray ionization trap mass spectrometry, *Rapid Commun. Mass Spectrom.*, 12, 361–367, 1998.

- Renault, J.-H., Thépenier, P., Zèches-Hanrot, M., Le Mon Olivier, L., Durand, A., Foucault, A., and Margraff, R., Preparative separation of anthocyanins by gradient elution centrifugal partition chromatography, *J. Chromatogr. A*, 763, 345–352, 1997.
- Revilla, E., Ryan, J.-M., and Martin-Ortega, G., Comparison of several procedures used for the extraction of anthocyanins from red grapes, *J. Agric. Food Chem.*, 46, 4592–4597, 1998.
- Ribéreau-Gayon, P., *University Reviews in Botany, Vol. 3, Plant Phenolics*, Heywood, V.H., Ed., Oliver and Boyd, Edinburgh, 1972.
- Rodriguez-Sanona L.E., Giusti, M.M., and Wrolstad, R.E., Anthocyanin pigment composition of red-fleshed potatoes, *J. Food. Sci.*, 63, 458–465, 1998.
- Saito, N., Abe, K., Honda, T., Timberlake, C.F., and Bridle, P., Acylated delphinidin glucosides and flavonols from *Clitoria ternatea*, *Phytochemistry*, 24, 1583–1586, 1985.
- Saito, N., Tatsuzawa, F., Yoda, K., Yokoi, M., Kasahara, K., Iida, S., Shighara, A., and Honda, T., Acylated cyanidin glycosides in the violet-blue flowers of *Ipomoea purpurea*, *Phytochemistry*, 40, 1283–1289, 1995.
- Saito, N., Timberlake, C.F., Tucknott, O.G., and Lewis, I.A.S., Fast atom bombardment mass spectrometry of the anthocyanins violanin and platyconin, *Phytochemistry*, 22, 1007–1009, 1983.
- Strack, D., Busch, E., Wray, V., Grotjahn, L., and Klein, E., Cyanidin 3-oxalylglucoside in orchids, *Z. Naturforsch.*, 41c, 707–711, 1986.
- Strack, D. and Wray, V., Anthocyanins, in *Methods in Plant Biochemistry*, Dey, P.M. and Harborne, J.B., Eds., Academic Press, New York, pp. 325–356, 1989.
- Strack, D. and Wray, V., The anthocyanins, in *The Flavonoids: Advances in Research Since 1986*, Harborne, J.B., Ed., Chapman & Hall, London, pp. 1–22, 1994.
- Sugui, J.A., Bonham, C., Lo, S.-C., Wood, K.V., and Nicholson, R.L., MALDI-TOF analysis of mixtures of 3-deoxyanthocyanidins and anthocyanins, *Phytochemistry*, 48, 1063–1066, 1998.
- Takeda, K., Harborne, J.B., and Self, R., Identification and distribution of malonated anthocyanins in plants of the Compositae, *Phytochemistry*, 25, 1337–1342, 1986.
- Takeoka, G.R., Dao, L.T., Full, G.H., Wong, R.Y., Harden, L.A., Edwards, R.H., and Berrios, J.D.J., Characterization of black bean (*Phaseolus vulgaris* L.) anthocyanins, *J. Agric. Food Chem.*, 45, 3395–3400, 1997.
- Torskangerpoll, K., Fossen, T., and Andersen, Ø.M., Anthocyanin pigments of tulips, *Phytochemistry*, 52, 1687–1692, 1999.
- Tsuda, T., Horio, F., and Osawa, T., Dietary cyanidin 3-*O*- β -D-glucoside increases *ex vivo* oxidation resistance of serum in rats, *Lipids*, 33, 583–588, 1998.
- Tsuda, T., Watanabe, M., Ohshima, K., Norinobu, S., Choi, S.-W., Kawakishi, S., and Osawa, T., Antioxidative activity of the anthocyanin pigments cyanidin 3-*O*- β -D-glucoside and cyanidin, *J. Agric. Food Chem.*, 42, 2407–2410, 1994.
- Van Sumere, C.F., Vande Castele, K., De Loose, R., and Heursel, J., Reversed phase HPLC analysis of flavonoids and the biochemical identification of cultivars of evergreen Azalea, in *Annual Proceedings of the Phytochemical Society of Europe*, Vol. 25, Van Sumere, C.F. and Lea, P.J., Eds., Clarendon Press, Oxford, U.K., 1985.
- Van Wyk, B.E. and Winter, P.J.D., Chemotaxonomic value of anthocyanins in *Podalyria* and *Virgilia* (Tribe Podalyrieae, Fabaceae), *Biochem. Syst. Ecol.*, 22, 813–818, 1994.
- Wang, H., Cao, G., and Prior, R.L., Oxygen radical absorbing capacity of anthocyanins, *J. Agric. Food Chem.*, 45, 304–309, 1997.
- Wang, H., Nair, M.G., Strasburg, G.M., Chang, Y.-C., Booren, A.M., Gray, J.I., and DeWitt, D.L., Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries, *J. Nat. Prod.*, 62, 294–296, 1999.

- Wang, J. and Sporns, P., Analysis of anthocyanins in red wine and fruit juice using MALDI-MS, *J. Agric. Food Chem.*, 47, 2009–2015, 1999.
- Wehr, T., Configuring HPLC systems for LC-MS, *LCGC*, 18, 406–416, 2000.
- Wilkinson, M., Swenny, J.G., and Iacobucci, G.A., High-pressure liquid chromatography of anthocyanins, *J. Chromatogr.*, 132, 349–351, 1977.
- Williams, D.H. and Fleming, I., *Spectroscopic Methods in Organic Chemistry*, 5th ed., McGraw-Hill, Berkshire, England, 1995.
- Wood, K.V., Bonham, C., Hipskind, J., and Nicholson, R.L., Analysis of anthocyanins and 3-deoxyanthocyanidins by plasma desorption mass spectrometry, *Phytochemistry*, 37, 557–560, 1994.
- Wood, K.V., Bonham, C.C., Ng, J., Hipskind, J., and Nicholson, R.L., Plasma desorption mass spectrometry of anthocyanidins, *Rapid Commun. Mass Spectrom.*, 7, 400–403, 1993.
- Wulf, L. and Nagel, C.W., HPLC separation of anthocyanins in *Vitis vinifera*, *Am. J. Enol. Vitic.*, 29, 42–49, 1978.

6 Amino Acid Analysis

Larry R. Massom

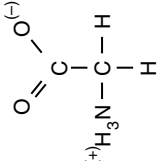
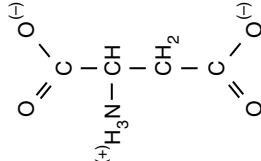
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6.1 INTRODUCTION

Although there are over 200 amino acids found in nature, genes (DNA) only contain codons for 20 amino acids (see Table 6.1 for a list of these amino acids and selected properties). These 20 amino acids are the foundation for all proteins, including all enzymes and structural protein. Some of these amino acids are then modified during and/or after the protein is synthesized. Other nonprotein amino acids are synthesized in the body. Many of the above-mentioned 200 amino acids do not occur in mammalian tissue but can be present in plant tissue. Some are intermediates in various

TABLE 6.1
Structures and Selected Properties of 20 Amino Acids

Type	Name 3-Letter Abbreviation Symbol	Structure	Molecular Weight	pKa	pI	Classification
	Glycine Gly G		75.07	2.34 9.60	6.0	Nonessential
	C ₂ H ₅ NO ₃					
	L-Aspartic Asp D					
	C ₄ H ₉ NO ₄					
Acidic			133.10	1.88 3.65 9.60	2.8	Nonessential

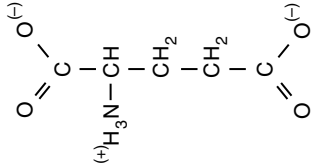
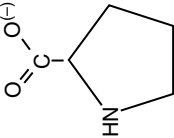
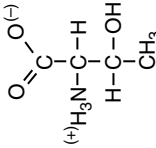
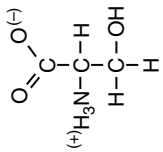
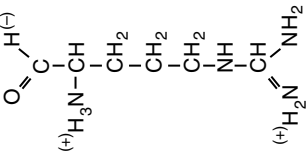
L-Glutamic (Glutamate) Glu E $C_5H_8NO_4$		147.13	2.19 4.25 9.67	3.2	Nonessential
Imino acid Proline Pro P $C_5H_9NO_2$		115.13	1.99 10.60	6.3	Nonessential
Hydroxyl Threonine Thr T $C_4H_9NO_3$		119.12	2.15 9.12	5.6	Essential

TABLE 6.1 (continued)
Structures and Selected Properties of 20 Amino Acids

Type	Name 3-Letter Abbreviation Symbol	Structure	Molecular Weight	pKa	pI	Classification
	Serine Ser S		105.09	2.21 9.15	5.7	Nonessential
	C ₃ H ₇ NO ₃					
Basic	L-Arginine Arg R		174.20	2.18 9.09 13.2	10.8	Semi-essential, required under certain metabolic conditions
	C ₆ H ₁₄ N ₄ O ₂					

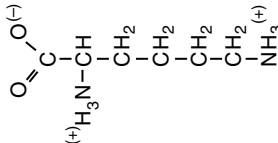
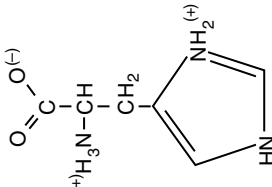
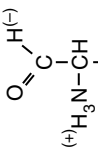
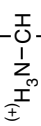
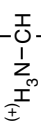
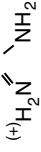
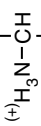
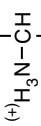
L-Lysine Lys K C ₆ H ₁₄ N ₃ O ₂		146.19	2.20 8.90 10.28	9.6	Essential
L-Histidine His H C ₆ H ₉ N ₃ O ₂		155.16	1.78 5.97 8.97	7.5	Essential for infants

TABLE 6.1 (continued)
Structures and Selected Properties of 20 Amino Acids

Type	Name 3-Letter Abbreviation Symbol	Structure	Molecular Weight	pKa	pI	Classification
Amide	L-Asparagine Asn		132.12	2.02 8.80	5.4	Nonessential
	D					
	C ₄ H ₈ N ₂ O ₃					
	L-Glutamine		146.15	2.17 9.13	5.7	Nonessential
	Gln					
	C ₅ H ₁₀ N ₂ O ₃					

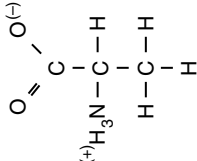
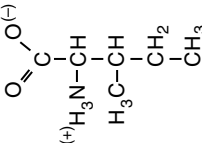
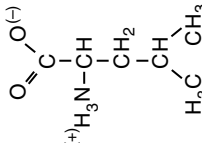
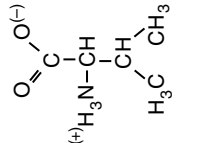
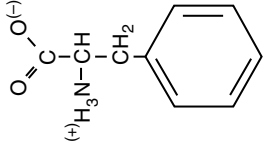
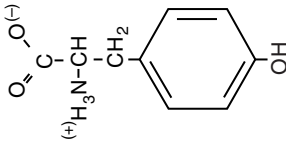
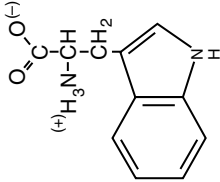
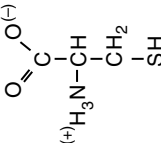
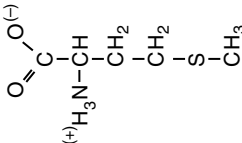
Aliphatic	L-Alanine Ala A $C_3H_7NO_2$		89.09	3.60 10.19	6.0	Nonessential
	L-Isoleucine Ile I $C_6H_{13}NO_2$		131.17	2.32 9.76	6.0	Essential
	L-Leucine Leu L $C_6H_{13}NO_2$		131.17	2.36 9.60	6.0	Essential
	L-Valine Val V $C_5H_{11}NO_2$		117.15	2.29 9.72	6.0	Essential

TABLE 6.1 (continued)
Structures and Selected Properties of 20 Amino Acids

Type	Name 3-Letter Abbreviation Symbol	Structure	Molecular Weight	pKa	pI	Classification
Aromatic	L-Phenylalanine Phe F C ₉ H ₁₁ NO ₂		165.19	2.58 9.24	5.5	Essential, can replace tyrosine
	L-Tyrosine Tyr Y C ₉ H ₁₁ NO ₃		181.19	2.20 9.11 10.07	5.7	Nonessential, can be replaced by phenylalanine

L-Tryptophan Try W $C_{11}H_{12}N_2O_2$		204.22	2.38 9.39	5.9	Essential
Sulfur containing					
L-Cysteine Cys C $C_3H_7NO_2S$		121.16	1.71 8.33 10.78	5.0	Nonessential, can partially replace the essential amino acid methionine
L-Methionine Met M $C_5H_{14}NO_2S$		149.21	2.28 9.21	5.7	Essential

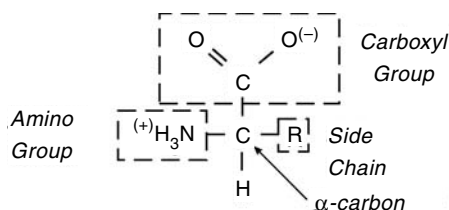


FIGURE 6.1 Generic structure of amino acids.

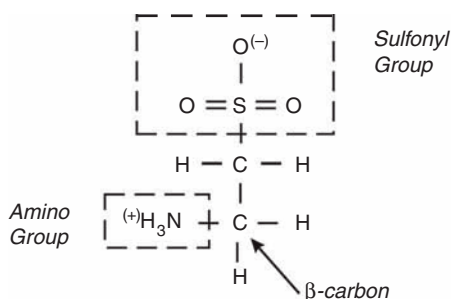


FIGURE 6.2 Taurine.

metabolic pathways; for example, ornithine is part of the urea cycle. Other nonprotein amino acids such as γ -aminobutyric acid (GABA) are neurotransmitters.

Structurally, the 20 amino acids found in proteins are α -amino carboxylic acids (see Figure 6.1), differing only in their side chains (R groups, see Table 6.1) with the exception of proline which is an α -imino, carboxylic acid. Some other nonprotein amino acids, however, only vary as to the location of the amine group, e.g., GABA, a γ -amine. Others may not even be a carboxylic acid; taurine, an amino acid important in the production of bile, is a β -amino sulfonic acid (see Figure 6.2)

Nutritionists have primarily focused on the 20 amino acids found in proteins, with special emphasis on the approximately 10 amino acids that are considered essential. Essential amino acids are those that must be present in a healthy diet because they cannot be synthesized by the human body. The exact list of amino acids that are considered essential varies slightly according to factors such as the age of the individual or the individual's state of health. For example, histidine is only essential in infants.

Since the focus has been on the ten essential amino acids, most nutritional laboratories have optimized their methods around these plus some of the other protein amino acids. Recently, nutritional supplements have begun to include some of the nonprotein amino acids such as ornithine in their formulations. These nonprotein amino acids may cause interference when analysis is performed by methods optimized for standard nutritional work. There are amino acid analytical methods developed for physiological matrices, which include many of the nonprotein amino acids. These methods may be adapted for nutritional matrices. There is also some interest

in non-physiological amino acids, e.g., 4-hydroxy-isoleucine, which are not found in mammalian tissue. The physiological methods will not include such amino acids, and specialized methods may need to be developed for these. It is important to know if a matrix contains these nonprotein amino acids; otherwise, they may either not be quantified by the method and/or interfere with the quantification of other amino acids.

Another important property of the protein amino acids is that they are all L-isomers, with the exception of glycine (glycine only has one configuration). The body cannot use the D-isomers of these amino acids, and, in fact, some D-isomers may be toxic. An exception is D-methionine, which the body can convert into L-methionine through transamination. Naturally produced amino acids are L-isomer; however, manufactured amino acids can be a racemic mixture with half of the molecules being D-isomers. These can then be separated and only the L-isomer used to fortify foods. The amino acid analysis methods normally employed in the nutritional laboratory will not differentiate the D- and L-isomers. There are, however, chiral chromatography and enzymatic methods that can be used if the product is suspected of containing D-isomers.

The amino acids in nutritional matrices may be present as free amino acids, peptides, proteins, and/or in polymers with nonpeptide bonds. Each of these forms may need to be prepared for analysis by different methods. Free amino acids can be directly extracted from the matrix and analyzed. Peptides and proteins must be hydrolyzed to free the amino acids before analysis. The analyses of the hydrolyzed matrices will give “total” amino acids, that is, those amino acids that were part of peptides/proteins as well as any free amino acids. The analysis of nonpeptide bonded polymers of amino acids such as aspartame often needs to be analyzed by specialized methods.

6.2 ACID HYDROLYSIS OF PROTEINS AND PEPTIDES

Acid hydrolysis is the primary method used to cleave the peptide bonds in proteins to release the amino acids for analysis. The conditions used in acid hydrolysis of foods for amino acid analysis are a set of compromises. These compromises are necessary because the stability of each amino acid varies, with threonine and serine being the most labile amino acids. The strength of the peptide bonds between the various amino acids also varies. Bonds involving valine, isoleucine, and leucine are the most difficult to hydrolyze. The compendium method (AOAC, 2000) using 6 *N* hydrochloric acid (HCl) at 110°C for 24 h is a method designed to minimize the degradation of the more labile amino acids while maximizing the release of all amino acids from the proteins. For most analytical work this will give reproducible, reliable results. There are, however, many pitfalls in this method that require experience and attention to detail. Many of these pitfalls are matrix and amino acid dependent. When more precise values are needed for the more difficult to hydrolyze or more labile amino acids, a time course of 6 *N* HCl hydrolysis can be conducted. Usually, the digestion times are 16, 24, and 72 h (Zumwalt et al., 1987). The values for the more labile amino acids such as threonine and serine are then determined by extrapolating

back to the zero time line. The value for the difficult-to-hydrolyze amino acids is determined by their maximum values. Time line analysis is not routine for most nutritional labs because of the expense and the delay in acquiring results.

For matrices that are not pure protein, the hydrolysis is usually done “wet.” The HCl and sample are together in the same vessel, usually a culture tube with a Teflon-lined cap or an ampule. A tight seal on the tube is important for two reasons. First, the presence of oxygen will degrade the amino acids and therefore needs to be minimized. With most food matrices, it is sufficient to either apply a vacuum or flush the tube with nitrogen and then quickly seal the tube. The competing hydrolysis of the carbohydrates and fats in the food matrix will quickly consume any remaining oxygen. Second, the tube needs to be sealed to prevent the loss of HCl. Pure HCl at room conditions is a gas. Concentrated HCl reagent (concentrated HCl) is the HCl gas dissolved in water to a concentration of approximately 37% or 12 *N*. The solubility of gases, unlike most solids, decreases as the temperature increases. This loss of HCl will result in incomplete hydrolysis, preferentially causing a low recovery in glutamic acid and serine. However, if the HCl loss is severe enough, all amino acid recoveries will suffer.

Most methods of acid hydrolysis agree that the acid should be HCl and that the concentration should be 6 *N*. Early researchers tried different concentrations and used different acids, but 6 *N* HCl gives the best recoveries. HCl is also easily removed from the hydrolysate by evaporation. This may be necessary for some of the pre-column derivatization methods. The amount of acid recommended is expressed in the molar ratio of acid to nitrogen content, with a ratio of 2800:1 being reported by Robel (1973) as being optimal. However, the matrix can affect the amount of acid required. A matrix high in soluble carbohydrates such as molasses may require a higher acid to nitrogen ratio (Roache and Gehrke, 1970).

Temperature and time are two interdependent variables that have been examined in detail by several researchers. Some of these researchers (Roache and Gehrke, 1970) have concluded that a temperature of 145°C for 6 h is a good alternative to the 110°C for 24 h used in compendial methods.

Protective agents such as phenol, 2-mercaptoethanol, and thioglycolic acid can be added to the hydrolysis step. It is believed that at least some of the tyrosine lost during acid hydrolysis may be due to its reaction with chlorine in the presence of any oxidizing agents. The addition of phenol, a halogen scavenger, may reduce this tyrosine loss. Bromine contamination will also result in the loss of tyrosine. The addition of reducing agents such as 2-mercaptoethanol and thioglycolic acid has been reported to also improve tyrosine recoveries. Inglis (1983) has reported that the addition of tryptamine increases the yield of tryptophan during acid hydrolysis.

Wet hydrolysis may not yield good results when using pure proteins, especially when the sample size is limited. Pure proteins can be hydrolyzed by “gas-phase hydrolysis” (Meltzer et al., 1987). With gas-phase hydrolysis, the HCl is placed in a separate container from the sample. The containers are then sealed into a secondary container. The secondary container is commonly a glass desiccator sealed with a Teflon gasket and placed in a desiccator cage. The desiccator is cycled between an inert gas, commonly nitrogen, and a vacuum, with the final cycle maintaining a

vacuum in the desiccator. The desiccator is then placed in an oven at 110°C for the appropriate time.

On heating, the HCl gas is released from the 6 *N* HCl, and the gas then hydrolyzes the protein. This is a good technique for pure proteins of limited quantity, since most contaminants present in the 6 *N* HCl are usually nonvolatile and therefore cannot contaminate the samples. Another advantage is that the HCl and moisture that may condense on the samples can easily be removed with vacuum, leaving the hydrolyzed protein in a state ready for precolumn derivatization or to be dissolved in the appropriate solvent for chromatography. The disadvantage is that some of the protective agents that can be added to the 6 *N* HCl are not volatile and are therefore ineffective with gas-phase hydrolysis.

Not all amino acids can be analyzed using only acid hydrolysis. Tryptophan is completely destroyed by acid hydrolysis, and therefore, the protein must be hydrolyzed using alkaline conditions. Glutamine and asparagine are completely converted to glutamic acid and aspartic acid during acid hydrolysis. Therefore, it must be considered that the glutamic acid and aspartic acid values include glutamine and asparagine. This is often denoted as Glx instead of Gln or Glu and Asx instead of Asn or Asp. Glutamine can be quantified if it is first converted to a compound that is stable during acid hydrolysis. Kuhn et al. (1996) report a method for pure proteins where the glutamine is converted to diaminobutyric acid by reaction with bis(1,1-trifluoroacetoxy)iodobenzene. The protein is then hydrolyzed and the diaminobutyric acid is quantified.

The sulfur-containing amino acids cysteine and methionine are partially degraded and/or oxidized during acid hydrolysis, especially in the presence of carbohydrate. Cysteine is partially converted (nonreproducibly) to cysteic acid, and methionine is partially converted (nonreproducibly) to methionine sulfoxide and methionine sulfone. These amino acids can be stabilized by completely oxidizing them to cysteic acid and methionine sulfone with performic acid before acid hydrolysis. This procedure will be discussed in more detail later.

6.3 ALKALINE HYDROLYSIS OF PROTEINS AND PEPTIDES FOR TRYPTOPHAN ANALYSIS

As has been stated, tryptophan is completely destroyed by acid hydrolysis. Some methods protect tryptophan with compounds such as 2-mercaptoethanol, but these methods do not always give reproducible results. Other methods for the liberation of tryptophan have included proteolysis by enzymes. An early method developed by Spies and Chambers (1949) did not require free tryptophan. It is a colorimetric assay, reacting the tryptophan with *p*-dimethylaminobenzaldehyde (PDBA). This method has been useful in pure proteins, but many food matrices have interfering substances. In addition, tryptophan is one of only two amino acids with a strong extinction coefficient in a usable ultraviolet (UV) range (approximately 280 nm depending on solvent, etc.) However, the most reliable method of tryptophan analysis is to release the amino acid from protein with alkaline hydrolysis (Lucas et al., 1980) and then use chromatography for quantification.

Several different alkalis have been used for the hydrolysis of tryptophan, including lithium, sodium, potassium, calcium, and barium hydroxides. Barium hydroxide has been reported to be less effective than either sodium hydroxide or lithium hydroxide. Also, the barium needs to be completely removed from the sample before chromatography. Usually, this is achieved by precipitation, which often reduces the recovery of tryptophan. Barium is also more toxic than the other cations. Potassium hydroxide and sodium hydroxide attack glass more vigorously than lithium or calcium hydroxide, but calcium hydroxide has solubility problems. Therefore, sodium and lithium hydroxides have become the most commonly used alkalis with very little difference in performance.

As with acid hydrolysis, alkaline hydrolysis is a compromise of maximizing the release of tryptophan while minimizing its degradation. If sodium hydroxide is being used, some researchers have reported less degradation if it contains 5% stannous chloride. The concentration of either sodium hydroxide or lithium hydroxide is usually 4 or 4.2 *M*. It is important to remove as much oxygen as practical by sonicating, vacuum, nitrogen flushing, or a combination of these techniques. The reaction is commonly carried out at 110°C for 20 h. Higher temperatures for shorter times have also been shown effective, such as 145°C for 4 to 8 h depending on the matrix (Roache and Gehrke, 1970).

The alkaline hydrolysis of tryptophan degrades many of the other amino acids; therefore, it is usually used solely for the analysis of tryptophan. Because of this, tryptophan's strong absorbance at 280 nm can be used for quantification, thereby eliminating pre- or postcolumn derivatization. Reversed-phase chromatography using UV detection is an excellent quantification method for tryptophan.

6.4 PERFORMIC ACID OXIDATION OF CYSTEINE AND METHIONINE

As has been mentioned, the sulfur-containing amino acids cysteine and methionine are partially and irreproducibly converted to cysteic acid, methionine sulfoxide, and methionine sulfone, respectively, during acid hydrolysis. The most often used technique for overcoming this difficulty is to quantitatively convert these amino acids to derivatives that are stable during acid hydrolysis. Performic acid will oxidize cysteine to cysteic acid and methionine to methionine sulfone, both of which are stable during acid hydrolysis (Moore, 1963; Zumwalt et al., 1987). Cystine, the disulfide dimer of cysteine is also oxidized to cysteic acid. Performic acid oxidation cleaves the disulfide and oxidizes the thiol groups to sulfonate groups yielding two cysteic acids. The quantification of cysteine, even in the free state, is difficult. The thiol groups of two cysteines are easily oxidized by oxygen from the air to produce cystine. Acid hydrolysis promotes this reaction. This led to the early discovery of cystine as an amino acid and only later was it realized that cystine was simply a dimer of cysteine. Cysteine is the amino acid that has a DNA (gene) codon and not cystine. Also, the proportion of cysteine that exists in a protein as cystine is often small.

The performic acid used in oxidation of the sulfur-containing amino acids is produced in the laboratory by reacting formic acid with hydrogen peroxide. One part of 30% hydrogen peroxide is mixed with nine parts of 88% formic acid. This mixture is cooled (the hydrogen peroxide and formic acid may be cooled before mixing) and allowed to react for 1 h. The 1-h reaction time will maximize the concentration of performic acid. The cold performic acid is then added to the sample and allowed to react for 16 h while being kept cold, i.e., a cold water bath, a refrigerator, or in a -20°C freezer.

After oxidation, the sample is then acid hydrolyzed, but first the excess performic acid must be removed. This can be accomplished in several ways: (1) the oxidized sample may be evaporated to dryness by vacuum and heat or (2) a reducing agent such as hydrogen bromide or sodium bisulfite can be added. The hydrogen bromide must be completely removed before acid hydrolysis. This can be accomplished by a vacuum. Because of this, it is often used to ensure the complete removal of performic acid when the oxidized sample is evaporated to dryness for other purposes. The sodium bisulfite does not need to be removed. The sulfur dioxide evolved from the neutralization of the performic acid with sodium bisulfite should be removed by degassing and nitrogen purging.

6.5 MICROWAVE HYDROLYSIS

The major drawback to the compendium acid hydrolysis method is the long (24 h) digestion time. This offsets the advances made in the “quantification” portion of the analysis by improved high-performance liquid chromatography (HPLC) methods. In 1987, Chen et al. were the first to report the use of microwave ovens to hydrolyze proteins. They performed wet hydrolysis of 0.2- to 0.5-mg samples of ribonuclease A and insulin B chain in 6 *N* HCl and a 1:1 propionic acid–12 *N* HCl mixture. The reaction vessels were customized Teflon vials with silicon septa and Teflon caps. Earlier work in microwave digestion had shown that glass vessels could not withstand the pressures generated during hydrolysis. Each vial was flushed with nitrogen and heated in a household microwave oven for 3 to 5 min. The recoveries were in good agreement with the theoretical values and those obtained by conventional hydrolysis (24 h, 6 *N* HCl at 110°C). Chen et al. (1987) were unable to measure the acid temperature and pressures generated in the digestion vessels. Others have shown typical acid temperature to be in the range of 130° to 175°C with pressures greater than 100 psi.

Several researchers (Engelhart, 1997) have investigated the use of microwaves to hydrolyze proteins since Chen et al. (1987). Their findings have indicated that microwave hydrolysis is successful using the same reagents as conventional hydrolysis. Various researchers have found that the reaction time required varies with the protein or peptide. The difficult-to-hydrolyze peptide bonds such as Val-Val, Ile-Ile, etc. require more time, in some cases 60 min or more. However, this is still far shorter than the time required for conventional acid hydrolysis. However, the more labile amino acids threonine and serine can show greater losses at the higher temperatures generated during microwave acid hydrolysis.

Under strictly controlled conditions, amino acids have also been shown to be at least as stable under microwave hydrolysis as with conventional hydrolysis. Degradation of amino acids during microwave hydrolysis appears to be equivalent to that which occurs during conventional hydrolysis. The degree of racemization seems to be significantly less with microwave hydrolysis.

In wet hydrolysis, specialized reaction vessels are required for microwave hydrolysis. The material used to construct these vessels must (1) be transparent to microwaves, (2) be able to resist strong acids or alkalis, (3) be thermally stable at elevated temperatures (typically 100° to 180°C), (4) be able to withstand pressures of well over 100 psi, and (5) not interact with the microwaves. The materials commonly used are fluoropolymers such as Teflon. The vessels need to have a mechanism to allow them to be evacuated and flushed with nitrogen. For safety reasons, a pressure-release mechanism should also be incorporated. A number of reaction vessels have exploded during microwave digestion. Every precaution needs to be taken to prevent explosions for the protection of the laboratory personnel and the lab. For gas-phase hydrolysis, open autosampler vials are commonly placed into a reaction vessel that has the same properties as described for wet hydrolysis.

As stated earlier, the conditions of microwave acid hydrolysis must be tightly controlled for good results. Temperature seems to be one of the most critical parameters in using microwave acid hydrolysis. If the temperature is too high, there is increased loss of the more labile amino acids; however, if the temperature is not high enough, there is incomplete hydrolysis. Because of the temperature sensitivity of the hydrolysis, the inexpensive household microwave oven will not give consistent results. Microwave acid hydrolysis of proteins requires a laboratory-grade microwave oven, which has temperature probes with fully automated temperature feedback control. The expense of such a microwave oven, along with the expense of specialized reactor vessels, requires a relatively high initial capital investment as compared to the compendium acid hydrolysis.

Microwave acid hydrolysis is not a panacea for protein hydrolysis. It has the same problems and pitfalls as the compendium hydrolysis. Its chief advantage is a much shorter hydrolysis time, allowing more rapid turnover of samples.

6.6 FREE AMINO ACID EXTRACTION

Amino acid fortification is usually done with pure free amino acids. Since the concentration of free amino acids is extremely low in most “naturally” occurring food matrices, extracting the free amino acids will verify the fortification.

The high solubility in water of most amino acids makes their extraction straightforward; however, it is advisable to have the extractant the same or similar to the initial mobile phase used in the chromatography. If precolumn derivatization is used, the extractant should be either the solvent needed for the precolumn derivatization or a solvent easily exchanged to the derivatization solvent.

A weak HCl solution is a good extractant for free amino acids. If the chromatography is ion exchange with postcolumn derivatization, the extracted amino acids can be chromatographed directly. The weak HCl solution will protonate the amino

acids so that they will have the correct charge for application to the ion-exchange column. If precolumn derivatization is used, the weak HCl can be removed by evaporation under vacuum and the sample redissolved in the solvent dictated by the precolumn derivatization.

Recently, there has been interest in fortification and supplementation with cystine. Research indicates that increased cystine intake is beneficial to people and animals under stress. Pure cystine, however, is not very soluble in aqueous extractants unless the pH is either below 2 or above 8. Cystine is soluble in a 1.0 *N* HCl solution, and as long as any dilutions keep the HCl concentration greater than 0.1 *N*, the pH should remain below 2. This pH range can be chromatographed directly with ion-exchange postcolumn reaction chromatography. Another difficulty with cystine is that it is not very reactive with o-phthalaldehyde (OPA, discussed later), a common postcolumn and precolumn derivative. However, ninhydrin (also, discussed later) will react well.

Monosodium glutamate (MSG) is the sodium salt of glutamic acid and therefore easily quantified as free glutamic acid. Of course, this yields total free glutamic acid and does not distinguish between glutamic acid added as MSG or in some other form.

6.7 INTERNAL STANDARDS AND CONTROLS

The processes of both acid and alkaline hydrolysis are prone to sample loss, not only because of the extreme conditions needed for hydrolysis, but also because of the number of manipulations required to prepare the sample for chromatography. Any method with this potential for sample loss should use a suitable internal standard.

An internal standard needs to be added as early as possible in the procedure so that the maximum number of steps can be monitored. For amino acid hydrolysis, this should be right after the sample is quantitatively aliquoted and definitely before hydrolysis or oxidation.

The internal standard should have chemical and physical properties as close to those of the analyte as possible. If these conditions are met, then anything that may affect the recovery of the analyte will be reflected by a proportional loss of the internal standard. Ideally, the recovery of the internal standard should be near 100%. If it is, the concentration of the analyte in the sample may be adjusted using the internal standard recovery data. Too great a loss of the internal standard should be an indication that the preparation of the sample was flawed and should be repeated.

Another factor that must be considered when selecting an internal standard is possible interference with the analyte. In the case of amino acid quantification with HPLC, the internal standard must not coelute with the other amino acids. The internal standard's relative retention time to the amino acids of interest is also important. If the amino acids being quantified are eluted near the beginning of the gradient, it is more efficient to have the internal standard elute also near the beginning. This can greatly reduce the length of the chromatography run, allowing more samples to be chromatographed and making the results available earlier.

TABLE 6.2
Partial List of Amino Acid Derivatization Methods

Method	Chromatography	Detection	Remarks
Postcolumn			
Ninhydrin	Ion-exchange column Mobile phase: pH and NaCl gradient, usually citrate either sodium or lithium	Visible: 570 nm for primary amines; 440 nm for secondary amines	Detects primary and secondary amines
OPA (<i>o</i> -phthalaldehyde)	Ion-exchange column Mobile phase: pH and NaCl gradient, usually citrate either sodium or lithium	Fluorescence: Ex λ 340 nm Em λ 455 nm	Will only detect secondary amines if first oxidized with sodium hypochlorite
Precolumn			
OPA (<i>o</i> -phthalaldehyde)	Column: reversed-phase C ₈ or C ₁₈	Fluorescence: Ex λ 340 nm Em λ 455 nm	Will only detect secondary amines if first oxidized with sodium hypochlorite
PITC (phenylisothiocyanate) (Pico-Tag [®] , Waters Corp.)	Column: reversed-phase C ₈ or C ₁₈	UV: 254 nm ($\lambda_{\text{max}} = 269 \text{ nm}$)	Detects primary and secondary amines
FMOC (9-fluornylethyl chloroformate)	Column: reversed-phase C ₈ or C ₁₈	Fluorescence: Ex λ 263 nm Em λ 313 nm	Detects primary and secondary amines
Dansyl chloride	Column: reversed-phase C ₈ or C ₁₈	Fluorescence: Ex λ 360 nm Em λ 470 nm	Detects primary and secondary amines
Dabsyl chloride	Column: reversed-phase C ₈ or C ₁₈	Visible: 436 nm	Detects primary and secondary amines

For amino acid analysis, the internal standard is usually any number of amino acids that either do not occur in nature or are not present in the matrices being analyzed. For acid hydrolysis, norleucine is probably the most widely used internal standard, but other amino acids such as β -alanine are also used (see Table 6.2). An internal standard commonly used for the alkaline digest of tryptophan is 5-methyl tryptophan.

The use of an internal standard does not eliminate the need for control samples; it only supplements the control data. Because the effectiveness of the hydrolysis is dependent on the matrix being analyzed, the control should be either the same matrix or a matrix with very similar properties. This means a general nutrition lab will need more than one control. In a lab that analyzes many different matrices, it is impossible to track a control for each matrix. Therefore, the lab needs to select controls that best reflect the range of matrices and protein content. Not every control needs to be

analyzed with each batch of samples as long as the similar samples are batched together with a similar control.

6.8 DETECTION OF AMINO ACIDS

Today, most amino acid analysis is conducted with HPLC. Gas chromatographic methods are less commonly used. The detection of amino acids, however, is not as straightforward as with other substances. Only tyrosine, tryptophan, and phenylalanine have significant UV absorption at useful wavelengths, and only tryptophan and tyrosine are fluorescent. This leaves either using a universal detection method, such as refractive index, light scattering, or very low UV wavelengths, or labeling the amino acids by derivatization with a substance that absorbs in the visible/UV wavelengths or fluoresces. The derivatization can be done either after elution from an HPLC column (postcolumn derivatization) or before HPLC separation (precursor derivatization). Both of these systems have their advantages and disadvantages.

6.8.1 POSTCOLUMN DERIVATIZATION

As stated, postcolumn derivatization labels the amino acids after elution from the HPLC column. Postcolumn derivatization involves introducing the derivatization reagents to the HPLC column eluant before the detector and providing the necessary conditions so that the reaction can proceed either to completion or reproducibly to a stage to allow adequate detection. The typical postcolumn reactor system (see Figure 6.3) consists of a pump(s) to deliver the derivatization reagent(s) into the column eluant, a reactor to provide the necessary mixing and time for the reaction to occur, and usually a heater for the reactor. The reactor in most cases is a length of tubing of the same internal diameter as the tubing leading from the column to the detector. The length (volume) of the tubing along with the flow rate controls the time the reaction has to occur. Since the postcolumn pressures are much less than precolumn pressures, the choice of material used is wider. Typically, the tubing is polypropylene, PEEK, or stainless steel. The material needs to be compatible with the reagents and thermally stable at the reactor temperature.

Commonly for postcolumn derivatization of amino acids, the separation is done by cation-exchange chromatography (discussed later). In this case, the separation of the individual amino acids is based solely on the properties of the amino acids. Another advantage of postcolumn derivatization is that there is much less sample preparation needed. Typically, the hydrolyte is only filtered and diluted with an appropriate solvent, either water or the initial mobile phase. The automation of the derivatization reduces the amount of analyst time. The disadvantages of postcolumn derivatization are (1) that the addition of reagents to the eluant stream can broaden the peaks and reduce resolution, (2) the high cost of the postcolumn reactor, and (3) that the choice of derivatization is restricted by the kinetics of the reaction and compatibility between the derivatization reagents and the mobile phases. For operations running relatively large numbers of samples, postcolumn derivatization is a good choice. The savings in analyst time can easily offset the cost of the postcolumn reactor.

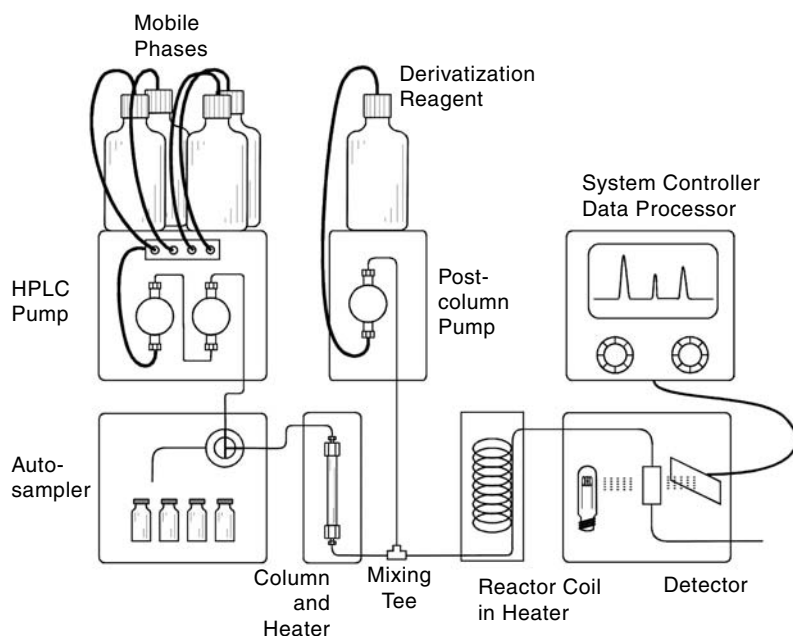


FIGURE 6.3 Chromatograph with a postcolumn reactor.

TABLE 6.3
Partial List of Internal
Standards

L-Norleucine
β -Alanine
L-Homoarginine hydrochloride
L-Norvaline
5- β -(4-Pyridylethyl)-DL-penicillamine
L-Homoserine

The two most commonly used postcolumn detection reagents are ninhydrin (Spackman et al., 1958) and OPA (Roth, 1971), with ninhydrin as the more commonly used (see Table 6.3 for a summary of these derivatizations).

6.8.1.1 Postcolumn Derivatization with Ninhydrin

The ninhydrin reagent contains two active components: ninhydrin and hydrindantin (reduced ninhydrin). Since ninhydrin, hydrindantin, and the reaction product Ruhemann's Purple are insoluble in water, an organic solvent that is miscible with water must also be present. Many ninhydrin formulations use DMSO (dimethyl sulfoxide). DMSO presents a hazard in the laboratory since it is readily absorbed through the skin and will act as a carrier for almost anything dissolved in it. Another solvent

used is methyl cellosolve. Methyl cellosolve is also absorbed through the skin and can produce severe headaches. Pickering Laboratories (Mountain View, CA) in its ninhydrin reagent TRIONE[®] uses Sulfolane[®] in place of DMSO.

The ninhydrin oxidizes the amino acid, releasing the amine group as ammonium, and is thereby reduced, forming hydrindantin. The hydrindantin then reacts with the liberated amine to form Ruhemann's Purple. The ninhydrin reagent contains additional hydrindantin to insure adequate concentrations to drive this reaction to completion. Ruhemann's Purple absorbs light at 570 nm. Secondary amino acids react differently. Again, the amino acid is oxidized to release the amine, which then reacts with the ninhydrin to form a yellow chromophore, which has a maximum absorbance at 440 nm. This allows the quantification of both primary and secondary amino acids, but requires the detector to either monitor two (2) wavelengths simultaneously or be able to switch between the two (2) wavelengths at the appropriate times.

The ninhydrin derivatization occurs very slowly at temperatures much below 120°C; therefore, the reactor must be heated. Typically, the reactor temperature is set between 125° and 135°C. The upper temperature approaches the boiling point of the reagents at typical reactor pressures. This may lead to precipitation of some of the components and blockage of tubing, including the detector flow cell. If the tubing or flow cell is completely blocked, it often cannot be unblocked and will usually need to be replaced.

Since the amino acid is destroyed during ninhydrin derivatization and forms the same reaction product regardless of the original amino acid (except for the secondary amino acids), ninhydrin derivatization will only work as a postcolumn method.

6.8.1.2 Postcolumn Derivatization with *o*-Phthalaldehyde

A very sensitive and specific postcolumn derivatization reagent is OPA. OPA reacts with primary amines in the presence of a reducing reagent such as 2-mercaptoethanol, producing a fluorescent substance with an excitation wavelength of 340 nm and an emission wavelength of 455 nm. The reaction occurs in an alkaline borate buffer in the range of pH 9 to 11. The most commonly used form of borate is the potassium salt; however, the sodium salt has also been used.

The postcolumn reactor flow rate is equal to the column mobile phase flow rate. The volume of the reaction coil is configured to give a reaction time of 20 to 30 sec. The reaction will proceed adequately at room temperature, but with some systems, a higher response may be obtained with an elevated reaction coil temperature. A temperature of 50° to 60°C will usually maximize the response. This temperature is far lower than the 120° to 130°C required for the ninhydrin derivatization and therefore should not cause problems. The reaction coil can be made of stainless steel, polypropylene, Teflon, or PEEK.

Two amino acids, cysteine and proline, give poor or no fluorescence with OPA derivatization. The very low fluorescence of cysteine-OPA will only be a problem with free cysteine or its dimer, cystine. Since cysteine can be converted to cysteic acid using performic acid oxidation, this drawback can be eliminated because cysteic acid-OPA has a strong fluorescence.

Since proline is a secondary amine, it cannot react with OPA unless its pyrrolidyl ring is first opened, producing a primary amine. This can be accomplished by

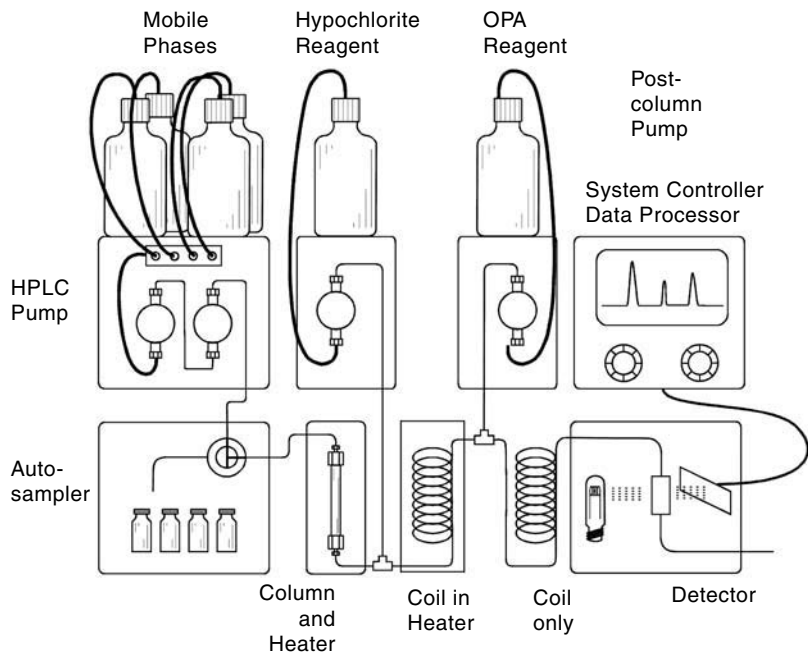


FIGURE 6.4 Chromatograph with a postcolumn reactor and postcolumn hypochlorite oxidation.

oxidizing proline with sodium hypochlorite (Dong et al., 1985). The sodium hypochlorite oxidation is done postcolumn using a second postcolumn reagent pump, mixing tee, and a heated reaction coil placed upstream of the OPA mixing tee (see Figure 6.4). A 0.0075% sodium hypochlorite solution in the borate buffer is pumped with a flow rate of one half of the column mobile phase and OPA reagent flow rate. The hypochlorite oxidation of proline occurs very slowly at room temperature. Therefore, the reaction coil must be heated to between 50 and 60°C. The volume of the reaction coil should allow a reaction time of 20 to 25 sec.

Lysine also yields lower fluorescence than the other amino acids. However, the addition of a detergent, typically Brij or sodium dodecyl sulfate (SDS), to the reaction will increase the signal.

Unlike ninhydrin, the reaction of amino acids with OPA does not destroy the amino acids, but adds the fluorescent tag to the primary amine. This allows OPA to also be used as a precolumn derivative, since the derivatization product is unique for each amino acid.

6.8.2 PRECOLUMN DERIVATIZATION

Precolumn derivatization has advantages and disadvantages as compared to postcolumn derivatization. The chromatography for most precolumn derivatization methods is commonly reversed phase using either C8 or C18 on silica. Silica supports can withstand higher pressures (usually up to 5000 psi) than the polymeric supports needed for ion-exchange chromatography used with postcolumn derivatization. This translates to higher flow rates and shorter run times.

The disadvantage of precolumn derivatization is increased manipulation of the sample before it can be chromatographed. Most autosamplers today can automate the derivatization reaction, thus freeing lab personnel for other activities. However, the derivatization reaction will usually not occur on the still very acidic hydrolyte. This necessitates the prior removal of the acid and water from the sample. This is commonly done by a vacuum. To completely remove the acid from the sample, the sample often has to be dried, redissolved (usually in water), and then dried a second time. Once dried, the sample is redissolved using a solvent compatible with the derivatization reaction. The additional time required by these steps can offset the savings achieved by using reversed-phase chromatography. Gas-phase hydrolysis of pure proteins eliminates the need to remove the acid from the sample.

With some precolumn derivatization methods, the derivatization reagents must also be removed before the sample can be chromatographed. This eliminates the automation of the reaction with the autosampler and increases the amount of sample preparation time.

6.8.2.1 o-Phthalaldehyde Precolumn Derivatization

OPA precolumn derivatization is probably the most commonly used precolumn method. OPA precolumn derivatized amino acids are detected with fluorescent detectors using the same wavelengths used for postcolumn OPA. The derivatized sample can also be loaded directly on the reversed-phase column, allowing automation of the derivatization reaction with an autosampler. The sample, however, does need to be evaporated to dryness after hydrolysis for removal of the acid. The removal of the acid needs to be complete, usually requiring redissolving and redrying.

The OPA derivatization reaction is rapid at room temperature, but, as with most chemical reactions, the time to “completion” can be long. This means that the derivatization reaction must be stopped at a carefully controlled time to get repeatable, accurate results. The reaction can be stopped either by injecting the sample onto the column or adding a stopping reagent to the reaction. Either of these means is applicable to autosampler automation (Carpino and Han, 1972).

The precolumn derivatization with OPA is conducted under similar conditions to the postcolumn derivatization condition. The reaction is carried out in a borate buffer with a pH of 9 to 11, containing Brij or SDS, and 2-mercaptaethanol. The reaction occurs at room temperature and can be stopped either by injecting the mixture onto the column, or by lowering the pH of the reaction by the addition of an appropriate buffer such as potassium dihydrogen phosphate with a pH around 4.3. This latter method is preferable since the results are easier to reproduce.

Precolumn derivatization with OPA has the same drawbacks as postcolumn derivatization. Secondary amino acids such as proline will not react unless first oxidized. Cysteine and cystine will not react, but can first be oxidized to cysteic acid. The addition of Brij or SDS to the reaction increases the fluorescence of lysine.

6.8.2.2 9-Fluorenylmethyl Chloroformate Derivatization

9-Fluorenylmethyl chloroformate (FMOC) (Carpino et al., 1972) is another popular precolumn derivatization reagent. FMOC derivatization produces a very stable

fluorescent product with both primary and secondary amines. The reaction is very straightforward. The sample is first mixed with a pH 7.7 borate buffer. Next the FMOC dissolved in acetone is added. The reaction is rapid, taking about 40 s.

The major drawback to the FMOC derivatization is that unlike OPA, FMOC is itself fluorescent and reacts with water to give a fluorescent product. If excess FMOC is not removed, it elutes in a broad band in the middle of the profile interfering with amino acid quantification. The excess reagent can be dealt with in two ways. A very hydrophobic amine such as 1-amino-adamantane can be added. This will then elute after lysine, the last amino acid to elute with reversed-phase chromatography. This, of course, lengthens the chromatography run time. The FMOC can also be extracted from the reaction by liquid–liquid extraction using pentane. This is usually done twice. The liquid–liquid extraction complicates automation of the reaction.

6.8.2.3 Other Precolumn Derivatization Methods

There have been many other precolumn derivatization methods developed, each with its own list of pros and cons. Table 6.3 lists some of the other derivatization methods. Some of these methods are available in commercial kits such as Waters Corporation's (Millford, MA) Pico-Tag[®] and AccQ-Tag[®]. These kits ensure quality reagents and very reproducible results. Commercial kits can be of benefit to the laboratory that does not conduct routine analysis of amino acids. However, these kits are not foolproof, nor do they guarantee good results. These kits may not work for all matrices, or the conditions may need modification with different matrices. No kit can completely replace experience.

6.9 CHROMATOGRAPHY OF AMINO ACIDS

The HPLC methods for amino acid analysis fall predominately into two types: ion exchange and reverse phase. Ion-exchange chromatography (IEC) is predominately used with postcolumn derivatization, while reversed-phase chromatography (RPC) is used with precolumn derivatization.

6.9.1 ION-EXCHANGE CHROMATOGRAPHY

The IEC used for amino acid analysis is strong cation exchange (scx), that is, the stationary phase has a negative charge. This negative charge attracts positively charged cations and repels negatively charged anions. The carboxyl and amine groups on amino acids are a weak acid and a weak base, respectively. Under acidic conditions (pH very much below 7), both the carboxyl group and the amine group will be protonated. This means that the carboxyl group will have a neutral charge ($-\text{COOH}$) and the amine will have a positive charge ($-\text{NH}_3^+$). If there are no ionizable side chains, the net charge of the amino acid will be positive and retained by the scx support.

The pH at which ionizable functional groups are completely protonated can be predicted from each group's pKa. The pKa is the pH at which, statistically, one half of the groups will be protonated. The pKa is not just a property of the ion alone, but is affected by neighboring structures. This means the pKa of the alpha carboxyl and amino group will vary from amino acid to amino acid (see Table 6.1 for the

pKa's of the 20 amino acids). In general, the pKa of the alpha carboxyl is around 2.3, and for the alpha-carbon amine about 9.4. For practical purposes, an ion can be considered completely protonated when the pH is 1.5 units below its pKa and deprotonated at 1.5 pH units above its pKa. At a pH around 3, most of the α -carbon carboxyls and α -amines will be protonated. This will result in the amino acid having a net positive charge, which in turn will cause the amino acid to charge pair with the scx support. As the pH of the mobile phase is increased, however, the carboxyl and amines will lose protons. The carboxyl groups will become negative ($-\text{COO}^-$) and the amines will become less positive, giving the amino acid a net negative charge. This will result in the scx support repelling (eluting) the amino acids.

As can be seen in Table 6.1, however, some amino acids have ionizable side chains with their own pKa. This obviously will affect the net charge of the amino acids. As the mobile phase goes from very acidic to more basic, the acidic amino acids (carboxyl group as part of their side chain) will gain a net negative charge at low pH and will elute first. The basic amino acids will retain a net positive charge until a much higher pH is obtained and will elute last. The elution order of amino acids can be predicted (with a few exceptions) by the pH at which its net charge is neutral (see Figure 6.5). This pH of the net neutral charge is known as the isoelectric point and is quantified as pI (see Table 6.1).

As stated, the pIs of the amino acids only give an estimate of the elution order. The other factor which affects elution order is the hydrophobicity of the amino acids. The scx support used in amino acid analysis is usually the sulfonated styrene. The use of styrene instead of silica is necessary because of the pH range of the mobile phases needed for amino acid separation. The pH range can be from pH 2.7 to over pH 11 for the column-regenerating mobile phases used to strip any ionic compounds from the column. Silica supports are not stable when the pH of the mobile phases fall much below 3 or above 7.5. When the pH is near 2, the covalent bond attaching the bonded phase will begin to be hydrolyzed, resulting in the degradation of the support. When the pH is above 7.5, the alkaline condition will begin dissolving the silica. Styrene is hydrophobic and therefore will interact with the hydrophobic portion of the amino acid structure. Having two modes of separation is referred to as being "mixed mode." Under most circumstances, mixed mode chromatography is considered undesirable since it complicates method development. For amino acid chromatography, the mixed mode aids in resolving the more hydrophobic amino acids that elute in the center of the profile.

Most IEC methods for amino acids use citrate as a buffering agent at a concentration of 0.2 M. Citrate has pKas of 3.14, 4.77, and 6.39; therefore, citrate works well, having a buffering capacity down to about pH 2.7 and up to about pH 7.5. pH 7.5 is still below the pIs of several amino acids (see Table 6.1). If a pH gradient is the only means of elution, the elution time of basic amino acids will be extremely long. To reduce the elution times of the basic amino acids, a salt gradient occurs simultaneously. The initial concentration of the salt cation is 0.2 N, with the final concentration reaching 1.2 N. The cation competes with the amino acids for the negatively charged sites on the scx support. As the cation concentration increases, it becomes more competitive, eluting the amino acids. Sodium and lithium are used as the cation, with chlorine being the salt's anion. Research has shown that sodium

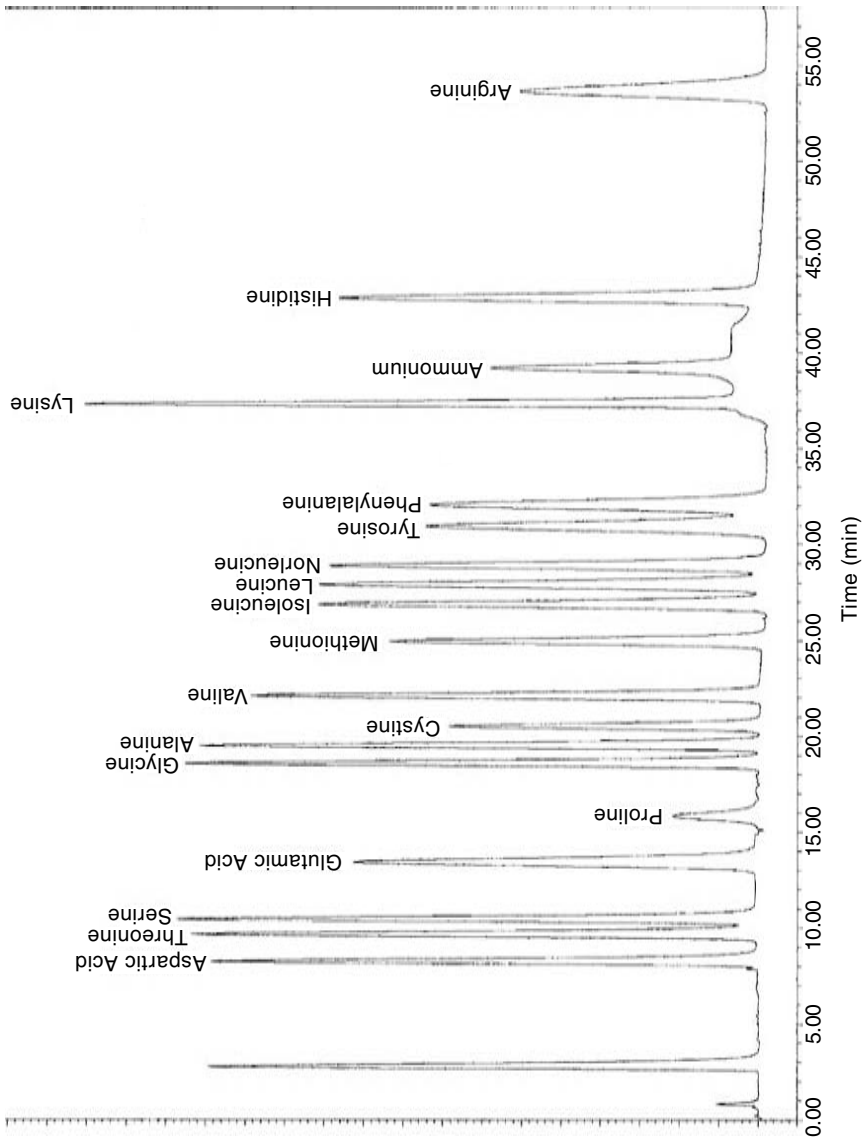


FIGURE 6.5 Chromatogram of ion-exchange separation of amino acids with postcolumn ninhydrin derivatization.

works best for most matrices; however, lithium works better for physiological fluids. The lithium cation competes more strongly than the sodium cation. This provides better resolution of some amino acids that are impossible to resolve with the sodium cations. The scx needs to be equilibrated with whichever cation is being used, so the columns are often sold for either sodium mobile phases or lithium mobile phases. A note of caution: the chloride ion will react with stainless steel. Since most HPLCs contain stainless steel fittings and tubing, this can cause some problems with corrosion.

Since citrate loses its buffering action above pH 7.5, some mobile phase systems use the borate ion as the final mobile phase buffering agent. Borate has pKas of 9.14, 12.74, and 13.8, making it an excellent buffer at very high pHs, but has no buffering action at neutral or acidic pHs. The change in ions from citrate to borate can cause baseline problems. Pierce Chemical Co. (Rockford, IL) manufactures a proprietary buffer (Buffelute[®]) system which is citrate free.

Many of the gradients used, especially early on, were step gradients. A step gradient occurs when the switch from one mobile phase to the next is abrupt. Step gradients are easier to automate, requiring simple switching at the appropriate time. A drawback of step gradients is that a series of mobile phases with small increments in pH and salt concentration must be made. With a step gradient, a minimum number of distinct mobile phases used is three, with six or more being typical. With the advent of cheap computers, continuous gradients can be accurately and repeatedly mixed by modern HPLCs. This allows reducing the number of different mobile phase down to as few as two.

Other substances are often retained on the scx support that cannot be eluted with 1.2 *N* sodium (or lithium) chloride at pHs of 7.5 or 8. Divalent cations such as calcium can be especially hard to remove and will “poison” the support, making it unusable. Therefore, it is common practice to “regenerate” the column either after every sample or on a routine basis. The most common regeneration mobile phase is 0.2 *N* sodium or lithium hydroxide. Many regeneration mobile phases will also contain a divalent chelater, usually EDTA [(ethylenedinitrilo)-tetraacetic acid].

The scx support for amino acid chromatography is a polymer, usually styrene. Polymers cannot withstand pressures as high as silica-based supports. Most polymers should not be subject to pressures over 2000 psi. If the support is subjected to too high of a pressure, it will be “crushed,” forming fine particles. The fine particles will migrate down the column, with the mobile phase becoming trapped in the frit at the end of the column. A column frit is a porous disk of stainless steel or a polymer used to retain the support in the column, but allowing the mobile phase to pass through. The pore size of the frit is typically 0.2 to 0.5 μm . The intact support cannot enter these pores, but the crushed support fines can and will usually become lodged. This will restrict the flow of the mobile phase, causing high pressures. A good diagnostic for this is to reverse the mobile phase flow through the column. The pressure will then drop for a while, but when the fines have migrated to the frit at the other end of the column, the frit will plug up and the pressure will increase again. The crushed support also has less volume than the intact support. This creates voids in the column, which will broaden the peaks and thus reduce resolution.

This lower pressure limit for polymeric supports translates into lower flow rates, typically below 0.5 ml/min, causing long run times. Grunau et al. (1992) show an

elution of 99 amino acids using the Pickering lithium buffers and column. A 135-min gradient was required to resolve all 99 amino acids. This is extreme, and for most nutritional applications the gradient with a regenerating cycle will take a little over 1 h. However, the Grunau et al. (1992) paper is a good reference for the relative elution time of many amino acids.

The column temperature also affects the retention time. The shift (shortening) in retention time due to increasing column temperature is significantly different for each amino acid. This makes column temperature an effective tool in improving resolution. The difference in effect of temperature on retention time can be used to shift the order of elution with some amino acid pairs. In addition, the column pressure is also inversely affected by temperature. The shorter retention time with the decrease in pressure favors using an elevated temperature. Typically, for IEC of amino acids the column temperature will range from 40° to 70°C.

Systems vary in important characteristics such as dead volume, which can greatly affect how closely the true gradient follows the program gradient. Because of the (dead) volume of the system, the true gradient will always lag behind the program gradient. Some of the older systems had a large dead volume due to the need for large pulse dampeners. The lag between the true and program gradient at the low flow rates needed for IEC could greatly extend the run time of the gradient. Also, column heaters are not all alike. Even though the true temperature of two column heaters may be identical, the configuration will affect the internal temperature of the column. Factors such as these are the reason that various parameters such as column temperature and gradient must be optimized for each system.

6.9.2 REVERSED-PHASE CHROMATOGRAPHY

Reversed-phase chromatography has a hydrophobic stationary phase and a hydrophilic mobile phase. The stationary phase most often is a porous silica bead with an alkyl-bonded phase. The diameters of the silica beads typically are 5 or 10 μm ; however, recently, smaller bead diameters of 2 and 3 μm are gaining in use. The pore size typically ranges from 60 to 300 Å. The silica support can withstand much higher pressures than the polymer supports, typically up to 5000 psi. This tolerance of higher pressure allows faster flow rates and, therefore, shorter retention times, speeding up the chromatography. But, as stated earlier, a silica support can only tolerate a pH range of 2 to 7.5.

The principle behind reversed-phase chromatography is the partitioning of the sample components between the hydrophobic stationary phase and the hydrophilic mobile phase. This is the same principle as liquid–liquid extraction. Whether or not the sample components are more attracted to the stationary phase or mobile phase depends on the components' relative solubility in hydrophobic and hydrophilic solvents. The relative amount of time the component spends in each phase determines the component's retention time. The hydrophobic components will have longer retention times.

The proportion of time that a component spends in each phase can be adjusted by changing the hydrophobic/hydrophilic characteristics of the two phases. The selections and proportions of its constituents adjust the hydrophobicity of the mobile phase. Decreasing or eliminating water from the mobile phase makes it more hydrophobic.

Using increasingly more hydrophobic organic solvents in the mobile phase will also increase its hydrophobicity. The most typical organic solvents used for amino acid chromatography are methanol and/or acetonitrile, which are fairly hydrophilic. Tetrahydrofuran (THF) is used less often and usually only as a portion of the organic constituent of the mobile phase.

The hydrophobicity of the stationary phase can be controlled by the selection of the alkyl chain bonded to the silica bead. The longer the alkyl chain is, the more hydrophobic the stationary phase. Octadecylsilane (ODS, C18) is the most commonly used support, but C8 is also used. A bead diameter of 5 μm is popular, but the use of 2- or 3- μm beads is increasing. The pore size for amino acids is not as critical as with larger molecules, and 60 Å or so will work fine.

The coating of the silica with the alkane is never complete, leaving silanol groups on the bead surface. Silanols are acidic (negatively charged) and will interact with protonated bases (positively charged), resulting in mixed mode chromatography as discussed earlier. This means that the pH of the mobile phase will affect the elutions of the amino acids. The mobile phase is therefore buffered. The most common buffer is acetate, and the pH is generally slightly acid or neutral. Remember, a high pH mobile phase (greater than pH 7.5) will dissolve the silica support and must be avoided.

In general, the order of elution for the amino acids is the following: the acidic and polar amino acids elute first, followed by the amino acids with short alkyl side chains, and finally the more hydrophobic amino acids (see Figure 6.6). Varying the mobile phase pH, the amount and type of organic solvent, and its ionic strength will affect the relative retention times of the amino acids. Adjustments of these parameters are necessary to optimize the chromatography. Jarrett et al. (1986) studied the affects of these parameters for OPA precolumn derivatized amino acids on C₈ silica. This study is an excellent guide to predict which parameter modification will improve the resolution of the various amino acids. Because of the small size of an amino acid molecule, the derivatization reagent will have an affect on the chromatography. Therefore, changing the various parameters will have different effects depending on which derivatization method is employed.

6.10 SUMMARY

Our understanding of the nutritional role of amino acids is rapidly expanding. Not only must the essential amino acids be supplied in the diet for good health, but it is being discovered that dietary intake of nonessential amino acids can improve our health and our ability to deal with and recover from stress. The sources of stress not only include illness and injury, but also exercise and fast-paced, high-pressure modern living. Today's health consciousness demands foods and supplements that not only contain the dietary requirements of the essential amino acids, but other amino acids as well. Because of this rapid expansion in understanding the importance of amino acids, health conscious consumers are demanding new products containing these amino acids. To meet this demand, producers and suppliers are rapidly providing these products. The amino acids composition of foods not only varies from food to food, but also within any single food due to different plant varieties, soils, weather, agricultural practices, processing, etc.

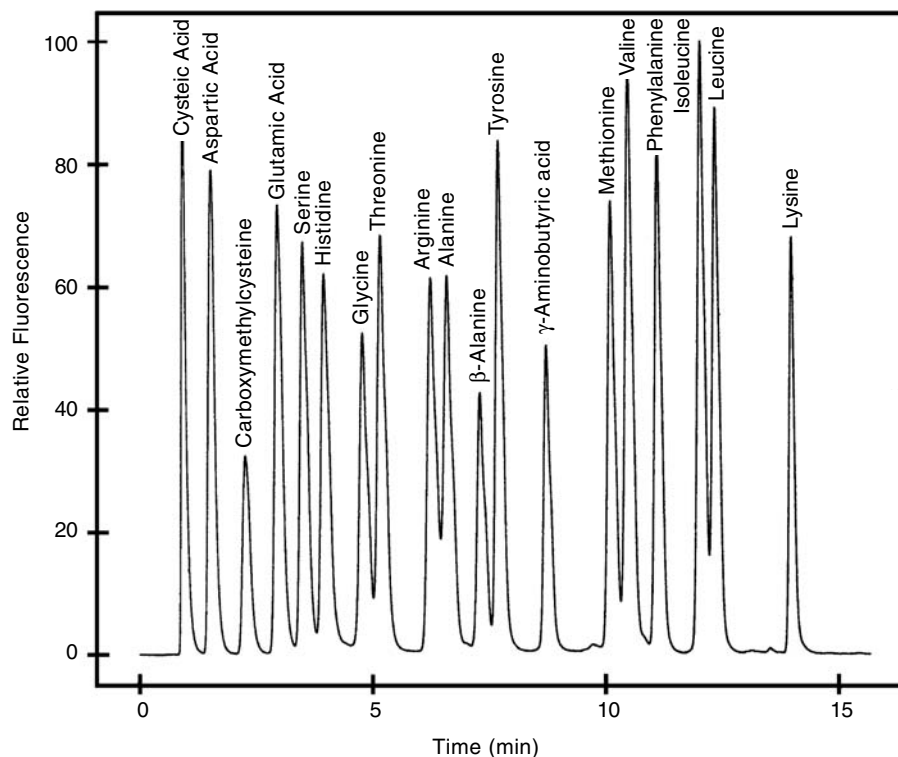


FIGURE 6.6 Chromatogram of reversed-phase separation of amino acids with precolumn OPA derivatization.

Fortification with pure amino acids also has variables. Therefore, laboratory analysis of foods and supplements is essential.

The analysis of amino acids is a complex issue. The first step in analyzing amino acids in proteins (which are most of the amino acids found in nature) is to release the amino acids to the free state by hydrolysis. Unless great expense and effort is expended, the conditions of hydrolysis must be a compromise to maximize amino acid release while minimizing amino acid degradation. Different matrices require different conditions. Time, temperature, amount of acid, presence of protective agents, etc. must be adjusted to meet the needs of the matrix.

Once the amino acids are free, they must be separated from each other and any other components of the sample. Today, HPLC is the most commonly used method of separation. The chromatography is usually ion-exchange chromatography (IEC) or reversed-phase chromatography (RPC). Amino acids IEC is almost exclusive on polymeric scx due to the use of the alkaline mobile phase. The buffering agent is predominately citrate. The RPC of amino acids is predominately done with a C8 or C18 silica stationary phase, with mobile phases buffered with acetate and containing either methanol or acetonitrile as the organic portion. Even though this appears to be standardized, there are still many variables which need to be optimized for each system to achieve adequate resolution of the amino acids included in the profile.

Once separated, the amino acids must then be quantified. The physical and chemical properties of amino acids, with the exception of tyrosine, tryptophan, and phenylalanine, limit detection to universal detectors such as refractive index (RI) or light scattering. The drawback of these detectors is that they are universal and any non-amino acid components will be detected, often interfering with the detection of the amino acids themselves. Derivatization coupled with either UV/visible or fluorescent detection is much more specific, eliminating the detection of most potentially interfering substances. For postcolumn derivatization, ninhydrin and OPA are used. There are a number of precolumn methods, including several commercially available kits. OPA is probably the most used precolumn derivatization method.

All the various methods for hydrolysis, chromatography, and derivatization, combined with the need to optimize these methods for the different matrices make amino acid analysis somewhat daunting. It is hoped that this chapter has served as an introduction and will aid in either the selection of a laboratory to conduct the analysis or as an aid to those who are beginning or conducting the analysis.

ACKNOWLEDGMENTS

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REFERENCES

- AOAC Official Method of Analysis, sec 982.30, Association of Official Analytical Chemists, Washington, D.C., 2000.
- Carpino, L.A. and Han, G.Y., The 9-fluorenylmethoxycarbonyl amino-protecting group, *J. Org. Chem.*, 37, 3404, 1972.
- Chen, S.T., Chu, S.H., and Wang, K.T., Rapid hydrolysis of proteins and peptides by means of microwave technology and its application to amino acid analysis, *Int. J. Pept. Protein Res.*, 30(4), 572, 1987.
- Dong, M.W. and Gant, J.R., High-speed liquid chromatographic analysis of amino acids by post-column sodium hypochlorite-*o*-phthalaldehyde reaction, *J. Chromatogr.*, 327, 17, 1985.
- Engelhart, W.G., Microwave hydrolysis of proteins and peptides for amino acid analysis, in *Microwave-Enhanced Chemistry*, Kingston, H.M. and Haswell, S.J., Eds., American Chemical Society, Washington, D.C., 1997.
- Grunau, J.A. and Swiader, J. M., Chromatography of 99 amino acids and other ninhydrin-reactive compounds in the Pickering lithium gradient system, *J. Chromatogr.*, 594, 165, 1992.
- Kuhn, K.S., Stehle, P., and Furst, P., Quantitative analyses of glutamine in peptides and proteins, *J. Agric. Chem.*, 44, 1808, 1996.
- Inglis, A.S., Single hydrolysis method for all amino acids, including cysteine and tryptophan, *Methods Enzymol.*, 91, 27, 1983.
- Jarret, H.W., Cooksy, K.D., Ellis, B., and Anderson, J.M., The separation of *o*-phthalaldehyde derivatives of amino acids by reversed-phase chromatography on octylsilica columns, *Anal. Biochem.*, 153, 189, 1986.

- Lucas, B. and Sotelo, A., Effect of different alkalies, temperature, and hydrolysis times on tryptophan determination of pure proteins and of foods, *Anal. Biochem.*, 109, 192, 1980.
- Meltzer, N.M., Tous, G.I., Gruber, S., and Stein, S., Gas-phase hydrolysis of proteins and peptides, *Anal. Biochem.*, 160, 236, 1987.
- Moore, S., On the determination of cystine as cysteic acid, *J. Biol. Chem.*, 238(1), 235, 1963.
- Roach, D. and Gehrke, C.W., The hydrolysis of proteins, *J. Chromatogr.*, 52, 393, 1970.
- Robel, E., Effect of the volume of HCl in minimizing amino acid losses when carbohydrate-containing samples are hydrolyzed, *Poultry Sci.*, 52, 604, 1973.
- Roth, M., Fluorescence reaction for amino acids, *Anal. Chem.*, 43(7), 880, 1971.
- Spackman, D.H., Stein, W.H., and Moore, S., *Anal. Chem.*, 30, 1190, 1958.
- Spies, J.R. and Chamber, D.C., Chemical determination of tryptophan in proteins, *Anal. Chem.*, 221, 1249, 1949.
- Zumwalt, R.W., Absheer, J.S., Kaiser, F.E., and Gehrke, C.W., Acid hydrolysis of proteins for chromatographic analysis of amino acids, *J. Assoc. Off. Anal. Chem.*, 70(1), 147, 1987.

7 Water-Soluble Vitamins

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7.1 INTRODUCTION

Vitamins are organic substances with very high biological activity that are required in small amounts for the growth and maintenance of human health. As they cannot be synthesized in the human body in the quantities required, they need to be supplied in the diet.

Although new trends in nutrition classify vitamins according to their metabolic functions, traditional classification based on their solubility is very useful in food science since they have similar natural sources and similar extraction procedures, mainly the fat-soluble vitamins.

In the last 20 years much attention has been paid to the determination of vitamins in foodstuffs due to their nutritional implications and the need for an adequate quality control of food products. Vitamin analysis in these matrices is difficult due to the high variability in their composition and the great number of components, which implies the risk of interference and the low vitamin content. Additional problems are the existence of different chemical compounds with vitamin effects and their stability influenced by light, heat, oxygen, or pH conditions. The development of new analytical techniques has led in recent years to analytical methods that are more rapid, accurate, and sensitive.

This chapter reviews the analytical techniques for water-soluble vitamins analysis, focusing on the main problems associated with each analytical methodology.

7.2 GENERAL CONSIDERATIONS ON VITAMIN ANALYSIS METHODOLOGY

As in all analyses, special attention needs to be paid to the sampling procedure in order to obtain a representative and homogeneous sample. Another critical point is to maintain the optimal stability conditions for each vitamin that guarantees lower vitamin losses over the analytical procedure; otherwise all the analyses are valueless.

Vitamin analytical methods can be classified into three categories:

1. Physicochemical methods are the most commonly employed and include the phases of extraction, purification, and final analysis. All these steps are critical in the vitamin evaluation. Extraction steps can include several treatments, such as heat, acid or alkali conditions, enzymes, and solvents, and these treatments have several purposes, such as vitamins stabilization and their release from other food components. Cleanup steps remove interfering compounds and are not necessary in many methods. Final determination and quantification can be mainly carried out by chromatographic [Table 7.1 summarizes some common high-performance liquid chromatography (HPLC) methods applied to water-soluble vitamin analysis], spectrometric, enzymatic, immunological, or radiometric techniques.
2. Microbiological methods use test microorganisms such as bacteria, protozoa, or yeast where growth is proportional to the presence of a specific vitamin. These microorganisms are cultivated in the presence of known quantities of the studied vitamin and in the presence of a food extract where the vitamin needs to be evaluated. The turbidity measurement and other parameters such as gravimetry, acid production, or gas production generally monitor the growth. An interesting related technique is the radiometric-microbiological assay based on the measurement of a ^{14}C -labeled metabolite, normally $^{14}\text{CO}_2$ formed by the test microorganism from a ^{14}C -labeled substrate.

Microbiological assays have the advantage of specificity and sensitivity, but they are highly time consuming if compared with the physicochemical methods and the analytical protocol must be strictly followed due to the variability in microbiological techniques.
3. Biological methods use laboratory animals. A control group is fed with a diet lacking in the studied vitamin and a second group is fed with the food in which the vitamin is being evaluated, or an extract of the food. Vitamin contents are measured by monitoring a physiological effect related to the vitamin intake. Ethical problems related to animal experimentation and the variability of these studies limit the use of biological methods to cases where no satisfactory methods are available.

The generalities of each vitamin with regard to structure, chemistry, stability, sources, bioavailability, metabolic functions, deficiency, and requirements are very well detailed in nutrition articles and treatises. For this chapter, we have summarized the structure, importance, stability, and sources according to the Merck Index (1989), Basu and Dickerson (1996), Thurnham et al. (2000), and Cuellar-Rodríguez (2000).

7.3 THIAMIN

7.3.1 GENERALITIES

Thiamin, also called aneurin and vitamin B₁, is present in most plant and animal tissues. A pyrimidine ring and a thiazole moiety linked by a methylene bridge are

TABLE 7.1
Representative HPLC Methods for Water-Soluble Vitamin Analysis

Vitamin	Sample Preparation	Stationary Phase	Mobile Phase	Detection	Matrix	Reference
Vitamin B ₁	Acid hydrolysis with 0.2 <i>N</i> H ₂ SO ₄ , autoclavation, enzymatic hydrolysis, filtration, oxidation with potassium ferricyanide, extraction with isobutyl alcohol	Lichrosorb RP-8	Methanol/acetone/nitrile/isobutyl alcohol (80:10:10)	FL 370:425 nm	Meat and vegetables	Bognar (1981)
Vitamin B ₁	Acid hydrolysis with 0.1 <i>M</i> H ₂ SO ₄ , enzymatic hydrolysis, centrifugation, oxidation	Nucleosil NH ₂ , 5 µm	25% K ₂ HPO ₄ buffer pH 4.4 in acetonitrile	FL 370:425 nm	Dietetic foods	Böttcher & Böttcher (1986)
Vitamin B ₁	Acid hydrolysis with 0.1 and 6 <i>M</i> HCl, autoclavation, enzymatic hydrolysis, Amberlite GC-50 and Sep-pack C ₁₈ clean-up	µ-Bondapak C ₁₈ , 10 µm	Several mixtures of methanol, acetic acid, hexane sulfonate and heptane sulfonate	UV 254 nm	Foodstuffs	Vidal-Valverde & Reche (1990a)
Vitamin B ₂	Acid hydrolysis with 0.1 <i>M</i> HCl, autoclavation, enzymatic hydrolysis, protein precipitation, Sep-pack C ₁₈ clean-up	Nucleosil C ₁₈ , 10 µm	0.01 <i>M</i> K ₃ PO ₄ buffer pH 7.0/acetone/nitrile (89.5:10.5)	UV 268 nm	Baby foods	Barná (1991)
Vitamin B ₂	Acid hydrolysis with 0.1 <i>M</i> HCl, autoclavation, enzymatic hydrolysis protein precipitation, filtration	Spherisorb ODS-2 5 µm	5 mM heptane sulfonic acid, pH 2.7/acetone/nitrile (3:1)	FL 227:520 nm	Cooked sausages	Valls et al. (1998)
Vitamins B ₁ and B ₂	Acid hydrolysis with 0.1 <i>N</i> H ₂ SO ₄ 10 min in boiling water, enzymatic hydrolysis, centrifugation, oxidation	µ-Bondapak C ₁₈	Methanol/water (60:40)	FL 360:415 nm	Cereal products	Mauro & Wetzel (1984)

Vitamin B ₃ -nicotinic acid	Acid hydrolysis with 0.1 and 6 <i>N</i> HCl, autoclavation, enzymatic hydrolysis, dilution, filtration and Douvex 1-X8 column purification	μ-Bondapak C ₁₈ , 10 μm	Methanol/0.01 <i>M</i> sodium acetate buffer pH 4.66 with 5 <i>mM</i> tetrabutylammonium	UV 254	Legumes and meats	Vidal-Valverde and Reche (1991)
Vitamin B ₃ -nicotinic acid and nicotinamide	Water extraction at 100°C, cooling and dilution	Partisil SCX, 10 μm	0.05 <i>M</i> K ₃ PO ₄ buffer, pH 3.0	UV 260 nm	Meat	Hamano et al. (1988)
Vitamin B ₃ -nicotinic acid and nicotinamide	Water extraction, centrifugation, deproteinization with zinc hydroxide, filtration	Spherisorb ODS-2	5 <i>mM</i> heptane sulfonic acid, pH 3.3/acetoneitrile (75:25)	UV 261 nm	Cooked sausages	Valls et al. (1999)
Vitamin B ₆	Extraction with HPO ₃ , centrifugation, filtration	BioSil ODS-5S C ₁₈	0.066 <i>M</i> H ₃ PO ₄ buffer, pH 3.0/acetoneitrile (90:10)	FL 290:395 nm	Meat	Ang et al. (1988)
Vitamin B ₆	Extraction with 5% TCA, dilution, centrifugation, enzymatic hydrolysis, protein precipitation, centrifugation, filtration	Hypersil ODS	Methanol/0.1 <i>M</i> KH ₂ PO ₄ buffer, pH 2.15 containing octanesulfonic acid (3:97)	FL 333:375 nm	Foods	Schoonhoven et al. (1994)
Biotin	37°C incubation of samples with 1% reduced glutathione solution, 1% EDTA solution, citrate buffer pH 5.7 and papain solution (in samples with high starch content, Takadiastase), filtration, postcolumn derivatization with avidin FITC	Lichrospher 100 RP-18	0.1 <i>M</i> H ₃ PO ₄ buffer, pH 6.0/methanol (81:19)	FL 490:520 nm	Foods	Alelí et al. (1999)

TABLE 7.1 (continued)
Representative HPLC Methods for Water-Soluble Vitamin Analysis

Vitamin	Sample Preparation	Stationary Phase	Mobile Phase	Detection	Matrix	Reference
Folates	Extraction with 0.1 M acetate buffer containing 1% of ascorbic acid, pH 4.5, nitrogen flush, strong anion-exchange column cleanup	Merck RP-18	8% Acetonitrile in acetic acid pH 2.3	FL 310:352 nm	Milk and blood samples	Wigertz and Jägerstad (1995)
Folates	Extraction with 0.15 M phosphate buffer pH 6.0 containing 2% sodium ascorbate and 0.1% mercaptoethanol, deconjugation with hog kidney deconjugase at pH 4.9, strong anion-exchange column cleanup	Hypersil ODS and Spherisorb ODS	Acetonitrile/H ₃ PO ₄ buffer, pH 2.2, gradient 9% acetonitrile to 24% acetonitrile within 8 min lag phase 4 min	FL 290:356 nm FL 360:460 nm UV 290 nm	Fish, meat, eggs, and dairy products	Vähteristö et al. (1997)
Vitamin B ₁₂	Extraction with HCl; preconcentration on an octadecylsilica cartridge	Octylsilica	Acetonitrile/aqueous ammonium phosphate solution, pH 3.0 (5:95 to 30:70)	Radioassay	Dairy products	Fie et al. (1994)
Vitamin B ₁₂	HLC-disk 25, polyvinylidene fluoride filtration for removing oily particles	Inertsil ODS	50 mM KH ₂ PO ₄ , pH 2.1/acetonitrile (90:10)	VIS 550 nm	Foods and biological samples	Iwase and Ono (1997)
Vitamin C	Ascorbic acid oxidation, 2,4-dinitrophenylhydrazine (DNPH) derivatization	Nucleosil Si, 3 μm	<i>n</i> -Hexane/ethyl acetate/acetic acid/2-propanol (40:30:10:20)	UV 495 nm	Foods	Kodaka et al. (1985)

Vitamin C	Extraction with 250 mM HClO ₄ and 5% trichloroacetic acid, centrifugation	Partisil 10 SAX	60 mM sodium acetate buffer, pH 4.6	Electrochemical detection 0.75 V vs. Ag/AgCl	Animal tissues	Carr et al. (1983)
Vitamin C	Water dilution	μ-Bondapak-NH ₂	Methanol/0.25% KH ₂ PO ₄ buffer pH, 3.5 (50:50)	UV 244 nm	Beverages	Dennison et al. (1981)
Vitamin C	Extraction with 0.1 M citric acid, EDTA 5 mM and <i>n</i> -hexane, centrifugation, filtration	Ultrasphere ODS C ₁₈	0.1 M NaH ₂ PO ₄ buffer pH 5.0 containing EDTA 5 mM and 5 mM tetrabutylammonium (50:50)	FL 350:430 nm	Foods	Vanderslice and Higgs (1988)
Vitamin C	Extraction with 80 g/l HPO ₃ and ethanol (35:65), centrifugation, 12 g/l NaSH solution addition and filtration	Cosmosil 5 C ₁₈	2 g/l PO ₃ H acid	UV 243 nm	Juices	Sawamura et al. (1990)

the constituents of the thiamin molecule. Thiamin diphosphate (TDP) and triphosphate (TTP) appear to be present in neural membranes. Thiamin is involved in the energy metabolism of proteins, lipids, and carbohydrates. This vitamin is also believed to have a role in nerve conduction and in some functions of the brain. Mostly in the liver and brain, thiamin is converted to its pyrophosphate form (TPP) to become biologically active as a coenzyme.

Thiamin is stable in slightly acid water up to the boiling point, but because of its water solubility, it can be leached out of food by boiling. This vitamin is unstable in alkaline solution. Thiamin is destroyed by ultraviolet (UV) irradiation. The loss of thiamin can be also enhanced by using rapidly boiling water in cooking vegetables, as the amount of oxygen in contact with the food increases. Thiaminases (thiamin-degrading enzymes) present in uncooked freshwater fish, shellfish, and some bacteria destroy thiamin by displacing the pyrimidine methylene group with a nitrogenous base or SH-compound. Furthermore, there also exist heat-stable anti-thiamin factors that generally bind to thiamin, rendering the vitamin biologically unavailable. These substances oxidize the thiazole ring to yield thiamin disulfide (which cannot be absorbed); among them, *o*- and *p*-hydroxy polyphenols of tea, betel nuts, many vegetables, and some animal tissue have been found. The primary dietary sources of thiamin are unrefined cereal grains or starchy roots and tubers; meat and meat products, especially pork, are also good thiamin sources. However, much of the dietary thiamin in developed countries appears in fortified cereals and breads, as well as in many functional foods.

7.3.2 ANALYTICAL METHODS

7.3.2.1 Physicochemical Determination

In these methods special care should be taken to protect samples and standards from light and heat at alkaline pH. The proposed methods are based on fluorimetry and HPLC.

- **Fluorimetric determination:** After extraction and enzymatic hydrolysis of the phosphate esters of thiamin, the vitamin is cleaned up by ion-exchange chromatography and oxidized to thiochrome by treating with potassium ferricyanide [$K_3Fe(CN)_6$] or other oxidant reagents such as $KMnO_4$, MnO_2 , $CNBr$, $HgCl_2$, and H_2O_2 . The thiochrome is finally extracted into an organic solvent such as isobutyl alcohol and analyzed by fluorimetry employing 360 to 365 nm as the excitation wavelength and 460 to 480 nm as the emission wavelength.
- **HPLC determination:** These are the most common techniques nowadays. In most food sample matrices, acid and/or enzyme treatment is needed to release thiamin from phosphate and proteins. Different acid conditions and enzymes have been proposed, including hydrochloric, trichloroacetic, and sulfuric acids; single enzymes such as papain and pepsin; or enzyme mixtures such as takadiastase or claradiastase. The last enzyme hydrolysis coupled to hydrochloric acid has proved to be the most efficient treatment.

Although some methods determine the B₁ vitamin directly by HPLC with UV detection, the low vitamin contents and the high quantity of interfering compounds in foodstuffs make its determination better by conversion of thiamin to fluorescent thiochrome and carrying out the final analysis by HPLC with fluorescence detection. This technique also increases the sensitivity and reproducibility. The thiochrome determination has two important problems: (1) the instability of the thiochrome itself, and (2) the chromatographic problems related to the excess of oxidizing reagent. The former problem can be solved by the addition of orthophosphoric acid which minimizes the formation of thiamin disulfide and stabilizes the thiochrome. The last problem can be solved by solid-phase chromatography cleanup using C₁₈ cartridges or by extracting the thiochrome into isobutyl alcohol as soon as it is formed. This can be achieved by adding the isobutyl alcohol before the ferricyanide reagent.

For the final analysis, reversed-phase HPLC offers the best results. Columns most commonly employed are C₁₈ and C₈, but others such as amino have also been employed. Mobile phases, including organic solvents, ion pair, and organic-aqueous buffer mixtures, have been used.

7.3.2.2 Microbiological Studies

Microbiological studies take from 4 to 72 h and detect thiamin amounts between 5 and 50 ng. The species most commonly employed for thiamin determination are *Phycomyces blakeslekanus*, *Kloeckera brevis* (ATCC 9774), *Ochromonas danica*, *Neurospora crassa*, and some Lactobacilli such as *Lactobacillus fermentum* and *L. viridescens* (ATCC 1270 C).

7.3.2.3 Biological Methods

Biological methods are hardly used, as they are time consuming (6 to 8 weeks) and poorly reproducible. These studies are based on the effect of thiamin on the growth and evolution of diseases related to vitamin B₁-lacking effects. Experimental animals include rats, pigeons, and chickens.

7.4 RIBOFLAVIN

7.4.1 GENERALITIES

Riboflavin, also called vitamin B₂, is structurally composed of an isoalloxazine ring with a ribityl side chain at the nitrogen at position 10. This vitamin functions metabolically as the essential component of two flavin coenzymes, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), complexed with proteins, which act as intermediaries in transfers of electrons in biological oxidation-reduction reactions. Both FAD and FMN function as coenzymes for flavoproteins of flavoenzymes. Flavoproteins are essential for the metabolism of carbohydrates, amino acids, and lipids and for pyridoxine and folate conversion to their respective coenzyme forms.

Even though riboflavin is described as water soluble, its solubility in water is actually very low but can be increased by the addition of urea and by formation of a complex with boron. It is insoluble in lipid solvents. Riboflavin is stable at increases

of temperature and in acid solutions. UV light causes irreversible decomposition of this vitamin. One of the most important problems of riboflavin analysis is the lability of flavins to light and alkaline conditions, so all analytical procedures have to be performed avoiding both factors. Acidification and enzyme hydrolytic treatment guarantee the complete hydrolysis of FAD and FMN to riboflavin. Riboflavin occurs in good amounts in dairy products. Animal protein sources such as meats, liver, kidney, and eggs are good sources of riboflavin, as well as some green leafy vegetables. Very good sources include yeast and meat extract.

7.4.2 ANALYTICAL METHODS

7.4.2.1 Physicochemical Determination

The extraction and cleanup steps for riboflavin are similar to those proposed for thiamin. Special care should be taken to protect samples and standards from UV light and alkaline conditions. Strict control in the oxidation process is also necessary to avoid riboflavin decomposition. Although riboflavin is classified as a water-soluble vitamin, some problems can arise when dissolving the vitamin in water, and this must be taken into consideration when preparing the standard solutions.

As in thiamin determination, proposed methods for riboflavin are based on fluorimetry and HPLC.

- **Fluorimetric determination:** After extraction and cleanup riboflavin fluorescence is measured employing 400 to 420 nm as the excitation wavelength and 550 to 570 nm as the emission wavelength. Despite its good sensitivity, this method has the disadvantage of interference from other fluorescent compounds.
- **HPLC determination:** Acid and/or enzyme treatments similar to those applied in thiamin analysis are normally required for the transformation of FMN and FAD to riboflavin. A few methods avoid this hydrolytic treatment, proposing direct dilution of the sample in water or acetate buffer followed by cleanup on a C₁₈ cartridge.

Final analysis is carried out by reversed-phase liquid chromatography on C₁₈, C₈, or C₂₂ columns, as well as by ion pair reversed-phase chromatography. Even though riboflavin, because of its structure, does not occur in ion form, the latter technique has proved to be very effective in separating riboflavin from disturbing components. Detectors used in both HPLC systems are mainly fluorescence or UV ones. Fluorescence excitation wavelengths range from 425 to 470 nm and emission wavelengths range from 510 to 570 nm. For UV detection 254, 268, and 270 nm are the selected wavelengths.

7.4.2.2 Microbiological Studies

The first assays used *Leuconostoc mesenteroides* (ATCC 9135) and *Tetrahymena pyriformis*, but *Lactobacillus casei* (ATCC 7469) is the microorganism most commonly employed nowadays in these studies.

7.4.2.3 Biological Methods

These studies are based on the biological responses of rats and chickens to riboflavin. They have been displaced by the previously described methods.

7.5 NIACIN

7.5.1 GENERALITIES

Niacin includes two vitamers, nicotinic acid and nicotinamide. Nicotinic acid is pyridine 3-carboxylic acid. It is slightly soluble in water and alcohol and easily soluble in alkalis, but is insoluble in both acetone and diethyl-ether. Nicotinamide is the active form, which functions as a constituent of two coenzymes: nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). In animal tissues, the reduced stages of these coenzymes have been found as the principal forms of niacin. Nicotinamide is very soluble in water, easily soluble in alcohol, soluble in glycerol, and slightly soluble in ether.

Niacin is categorized as a vitamin because its precursor, tryptophan, is an essential amino acid, so the human synthesis of niacin is dependent upon diets. Preformed niacin is widely distributed in plant and animal foods. The typical preformed niacin sources in diets are meat and meat products, cereals, dairy products, beverages, and eggs. However, cereals with esterified niacin in complexes have this vitamin unavailable for absorption, but its bioavailability can be increased by treatment with alkali to hydrolyze the esters. Coffee can be a source of niacin, as nicotinic acid is liberated in coffee by roasting.

7.5.2 ANALYTICAL METHODS

7.5.2.1 Physicochemical Determination

- **Spectrophotometric determination:** These methods require several extraction and cleanup steps for removing interfering compounds.

Final determination is based on the reaction between niacin and cyanogen bromide, which produces pyridinium compounds that finally react with aromatic amines, giving colored compounds. This method is hardly used nowadays because it does not distinguish between nicotinamide and nicotinic acid and it employs cyanogen bromide, which is a noxious and unstable reagent.

- **HPLC determination:** This technique has been used extensively for determining only one vitamer or both vitamers together. Simultaneous determination is difficult due to the differences in basicity and polarity of both vitamers and the interference problems. After water-phase extraction and sometimes cleanup steps as deproteinization, nicotinic acid and/or nicotinamide are analyzed by ion pair reversed-phase HPLC with UV detection. Most methods use different ion pair reagents for each vitamer, but a single ion pair reagent for nicotinamide and nicotinic acid can be also employed.

7.5.2.2 Microbiological Studies

Niacin microbiological determination uses some Lactobacilli such as *L. arabinosus* (ATCC 2112), *L. plantarum* (ATCC 8014), and *L. casei* (ATCC 7469), as well as *Leuconostoc mesenteroides* (ATCC 9135). *Leuconostoc mesenteroides* is not useful for nicotinamide determination. In general, growth is measured by turbidity, but acidimetric measurements can also be used. The latter determination requires longer incubation periods.

7.6 PYRIDOXINE

7.6.1 GENERALITIES

Vitamin B₆ is the generic term for 3-hydroxy-2-methyl-pyridine derivatives with the biological activity of pyridoxine. It occurs in three vitamers: pyridoxine (pyridoxol), pyridoxal, and pyridoxamine, in which alcohol, aldehyde, and amine groups, respectively, are located at the 4-position of the pyridine ring. All three vitamers are comparably active. Pyridoxal-5'-phosphate and sometimes pyridoxamine-5'-phosphate are the coenzyme forms of vitamin B₆. Pyridoxine is stable in acid solutions. It is rapidly destroyed by light. Pyridoxine is more stable than either pyridoxal or pyridoxamine. Vitamin B₆ in plants which contain essentially pyridoxine is lost less than the vitamin in animal foods which contain mostly pyridoxal and pyridoxamine. Many vitamin B₆-dependent enzymes are known, and almost all of them act on amino acids in reactions which include transamination, decarboxylation, dehydration, desulfhydration, racemization, cleavage, and synthesis, among other different processes.

No food is a particularly rich source of pyridoxine, as this vitamin is widely distributed in foods. Meats and cereals are the richest sources. In contrast, fruits are poor sources. In some vegetables, the form of vitamin B₆ is present as unavailable glucosides. In many foods the vitamin is bound to protein. If foods are stored for a long time (1 year), losses of vitamin B₆ may be important.

7.6.2 ANALYTICAL METHODS

7.6.2.1 Physicochemical Determination

- **Fluorimetric determination:** Even though B₆ vitamers exhibit native fluorescence, a previous purification and separation of vitamers by column chromatography is required for the final fluorimetric determination. For this reason HPLC methods are the most commonly used.
- **HPLC determination:** In many methods all six vitamers are extracted mainly in acidic conditions (trichloroacetic acid, metaphosphoric acid, or sulfosalicylic acid). Other methods hydrolyze the phosphorylated forms of the vitamin by H₂SO₄ in conjunction with autoclaving or by enzymatic treatment, thus determining only the nonphosphorylated vitamers.

Several HPLC systems have been used for the final analysis, including ion-exchange HPLC, reversed-phase HPLC, and ion pair reversed-phase HPLC. Detectors used in all HPLC systems are mainly fluorescence ones, although UV detectors have been sometimes used.

7.6.2.2 Microbiological Studies

The main problem related to vitamin B₆ microbiological determination is the different growth response to different vitamins. This problem can be solved by separating the vitamins by cation-exchange chromatography on a Dowex AG 50 W-X8 column. *Saccharomyces uvarum* (ATCC 9080) and *Kloeckera brevis* (ATCC 9774) are the test microorganisms most frequently used. When *S. uvarum* is employed, an additional problem can be the inhibition of growth due to sample extracts with high salt concentrations.

7.7 BIOTIN

7.7.1 GENERALITIES

Biotin is a sulfur-containing bicyclic compound in which tetrahydrothiophene and imidazolidone rings are fused and there is a valeric acid as the side chain. From the eight possible stereoisomers, only the dextrorotatory (D-(+)-biotin) is ordinarily found in nature and is the only one biologically active. This compound is sensitive to heat.

Biotin is widely distributed in nature in the free form or covalently bound to proteins or peptides. Good sources of biotin are organ meats, egg yolks, and milk.

7.7.2 ANALYTICAL METHODS

7.7.2.1 Physicochemical Determination

The proposed methods are based on spectrometric, chromatographic, and protein-binding techniques. As biotin occurs in food both in free form and covalently bound to proteins, in many methods it is necessary to break these bonds by acid or enzymatic hydrolysis. The former is more frequently used than the latter, since acid treatment converts D-biotin into D-biotin and allows a total determination of biotin. Enzymatic treatment with papain leaves D-biotin unchanged and only D-biotin is determined. Despite this, some methods propose enzymatic hydrolysis rather than acid hydrolysis because it does not induce any degradation of biotin.

- **Spectrophotometric determination:** The first spectrophotometric methods proposed for biotin quantification lacked adequate sensitivity and are hardly applied nowadays. The most interesting spectrophotometric method for biotin analysis is based on the reaction of biotin with periodate, resulting in iodate as the reaction product. This iodate reacts with iodide, forming triiodide which is finally measured at 350 to 352 nm. This method has a sensitivity down the microgram level.

- **Chromatographic determination:** Many chromatographic techniques have been proposed for biotin determination, including thin-layer chromatography, gas chromatography (after derivatization of biotin to silyl ester), and HPLC, which is the most common chromatographic technique in biotin analysis. Most proposed methods are reversed-phase HPLC on C₁₈ columns, but anion-exchange HPLC and normal-phase HPLC have been already used. Direct detection of the vitamin by UV is not sensitive enough due to the lower content of biotin in foodstuffs, thus a pre- or postcolumn derivatization into fluorescent derivatives is necessary to obtain a satisfactory detection limit (10^{-8} or 10^{-9} g ml⁻¹).
- **Protein-binding assays:** These techniques allow a rapid and sensitive determination of biotin and, therefore, are commonly employed for routine analysis of a great number of samples. In all these methods the binding protein is the avidin or the streptoavidin, using radioactive isotopes, enzymes, or chemiluminescent substances as labels. The disadvantage of these assays is their lack of discrimination between biotin and its metabolites. Combining protein-binding assays and HPLC can solve this problem.

7.7.2.2 Microbiological Studies

These methods were the first developed for biotin analysis and are still accepted as official methods because of their high sensitivity (nanogram level). The species most commonly employed for biotin determination are some Lactobacilli such as *L. arabinosus* (ATCC 2112), *L. plantarum* (ATCC 8014), and *L. casei* (ATCC 7469); *Ochromonas danica*, *Neurospora crassa*, and *Saccharomyces cerevisiae* (ATCC 4228) are also employed. *Kloekera brevis* (ATCC 9774) is useful for a radiometric-microbiological determination based on the measurement of ¹⁴CO₂ formed by the test microorganism from ¹⁴C-labeled L-methionine.

7.7.2.3 Biological Methods

These studies are based on the effect of biotin on the growth of rats or chickens, as well as on the determination of the activity of certain biotin-dependent enzymes such as pyruvate carboxylase.

7.8 FOLIC ACID

7.8.1 GENERALITIES

The folic acid molecule consists of a pteronic acid nucleus (a pterine molecule coupled to *p*-aminobenzoic acid) conjugated with one to seven molecules of glutamic acid. Because of this structural component, folic acid is also called pteroylglutamic acid. The pyrazine ring can also present different reduced forms such as dihydropteroylglutamic acid and tetrahydropteroylglutamic acid. Different single carbon substituent groups such as formyl, methyl, and methylene can be also attached to N⁵ and N¹⁰ positions of the pyrazine ring. This results in the existence of over 150 different

forms of this vitamin in nature which may differ in their bioavailability. All biologically active forms of folic acid are known as folacin(s) or folate(s).

Folic acid is synthesized by plant and certain microorganisms and passed on to higher animals through the food chain. Good sources of folate include liver, dark-green leafy vegetables, legumes, wheat germ, egg yolks, and yeast. Although the monoglutamate form is present in foods, most of the folates are in the form of polyglutamate which is important for their analysis, since in both physicochemical and microbiological analyses enzymatic deconjugation treatment is necessary to the hydrolyze polyglutamate to mono- or diglutamate forms. Folates are labile to changes in pH, the presence of oxidizing agents, and exposure to heat and light.

7.8.2 ANALYTICAL METHODS

7.8.2.1 Physicochemical Determination

- **HPLC determination:** The two main problems in the the development of successful HPLC methods for folates determination are the presence of many different forms of this vitamin and their low levels in foodstuffs. Additional problems are their instability to light, heat, and oxidation. For preventing oxidation losses of folate, certain antioxidants such as ascorbic acid or nitrogen flushing are employed during the extraction procedure.

After extraction and enzymatic deconjugation, sample extracts are cleaned up by strong anion-exchange chromatography. Final analysis is performed by reversed-phase HPLC with fluorescence or UV detection.

- **Protein-binding assays:** Several enzyme protein-binding assays and radioprotein-binding assays have also been developed and commercialized as kits for rapid evaluation of this vitamin in blood, plasma, and different foodstuffs. Despite their rapidity, these methods lack adequate discrimination between different forms of folates.

7.8.2.2 Microbiological Studies

Due to the difficulties in the estimation of total folates in food by physicochemical methods, official methods are based on microbiological assays. As in the previously described methods, special care must be taken to protect labile folates from oxidation and photochemical degradation. For evaluation of free folic acid, *Enterococcus hirae* and *Streptococcus faecalis* (ATCC 7830) can be used, but they do not respond to other folate forms present in food. The most widely accepted procedure for total folates determination uses *Lactobacillus casei* ssp. *rhannosus* (ATCC 7469) as the test microorganism, since this bacterium responds to most native folates present in food. The main problem involved with *Lactobacillus* is the presence of food-bound folates that do not promote the growth of these bacteria. It is known that the number of glutamyl residues linked to the pteroyl group is negatively related to the *L. casei* growing response. For this reason an enzymatic treatment of foods with folate conjugase is necessary to hydrolyze folylpolyglutamates to mono- or diglutamate

forms. Recent studies have found that treatments with protease and α -amylase improve the folate determination in high protein and high carbohydrate foods.

7.9 VITAMIN B₁₂

7.9.1 GENERALITIES

Vitamin B₁₂ is the name given to a group of active vitamers called cobalamins which contain cobalt. The structure of this vitamin consists of a macrocyclic ring (usually called “corrin”) formed by four reduced pyrrole rings linked together. Two of the four pyrrole rings are joined directly rather than through a single methylidyne carbon. Below the corrin ring system there is a 5,6-dimethyl-benzimidazole riboside that is linked with one end to the central cobalt atom. The form called cyanocobalamin contains a cyanide group coordinately bound to the cobalt atom of the vitamin B₁₂ molecule. The cyanide group may be removed and substituted by a hydroxy, aqua, nitro, methyl, or 5'-adenosyl group called “hydroxycobalamin,” “aquacobalamin,” “nitrocobalamin,” “methylcobalamin,” and “5'-adenosylcobalamin,” respectively.

Crystalline vitamin B₁₂ is stable to heating at 100°C. Aqueous solutions of vitamin B₁₂ at pH 4 to 7 can be autoclaved with very little loss. Cyanocobalamin is the most stable form. Nevertheless, the cobalamins are very sensitive in the presence of reducing agents, as well as in light (especially adenosyl- and methylcobalamins) and alkaline conditions. Photolysis results in two compounds stable only under anaerobic conditions.

In plant foods, vitamin B₁₂ cannot be detected, unless it is contaminated with microorganisms (which may occur in legumes and root vegetables). For humans, the most reliable sources of this vitamin are animal foods such as meats and seafood. Algae can also contain vitamin B₁₂, but the bioavailability has been questioned.

7.9.2 ANALYTICAL METHODS

7.9.2.1 Physicochemical Determination

- **Spectrophotometric determination:** Even though this technique has been used for vitamin B₁₂ determination, it is not a suitable method for a complex sample matrix.
- **HPLC determination:** This technique has been widely used, since, in general, it is less sensitive than the microbiological method. The various isomers of vitamin B₁₂ (cyanocobalamins) can be analyzed by reversed-phase HPLC. Pre- and postcolumn labeling, column switching, and solid-phase extraction (SPE) may be used (Iwase and Ono, 1997).
- **Protein-binding assays:** The **radioisotope dilution assay** (RIDA) has been employed for determining vitamin B₁₂ in foods, using radiolabeled B₁₂ and hog intrinsic factor (IF), the most specific B₁₂-binding protein. Several kits for the RIDA method are commercially available. A very good correlation coefficient has been found between the microbiological method and the RIDA method, but a radioisotope is used and radioisotope facilities and apparatuses are necessary for the RIDA method. The **direct**

competitive ELISA enzyme immunoassay (“Ridascreen[®]” test, Digen Ltd., U.K., 1998), developed in a microplate with a very specific vitamin B₁₂ antibody, can be used for vitamin B₁₂ quantification both in foods and in pharmaceuticals. This technique is very sensitive. In foods such as milk, 250 ng/l can be measured. The fat content of oily samples needs to be previously removed. Depending on the foodstuff analyzed, previous enzymatic treatment of the sample may be required. Vitamin B₁₂ can also be analyzed in foods with a fully automated chemiluminescence B₁₂ analyzer, with the acridinium ester-labeled B₁₂ derivative and IF. Vitamin B₁₂ is extracted from foods applying the method reported in the microbiological techniques paragraph, but, in the case of the chemiluminescence method, vitamin B₁₂ is extracted with distilled water from pieces of filter paper after 1 day at 4°C in the dark. Standards are also used. The compounds extracted from the filter papers are determined spectrophotometrically by measuring absorbance at 361 nm. The vitamin B₁₂ extracts are directly applied to the chemiluminescence B₁₂ analyzer. If compared with the microbiological method, the chemiluminescence method appears to be 5 to 10 times less sensitive, but simpler, quicker, highly selective, and reproducible (Watanabe et al., 1998).

7.9.2.2 Microbiological Studies

Microbiological studies can be used for determining vitamin B₁₂ in addition to radioisotopic techniques (Rougereau et al., 1997). The following strains are most often used: *Lactobacillus lactis* (ATCC 8000), *L. leishmannii* (ATCC 7830) (Schneider, 1987), and *Escherichia coli*, according to the classical methods of determination. The extraction of vitamin B₁₂ can be carried out by homogenizing the food samples in acetate buffer, followed by extraction of total vitamin B₁₂ from the homogenates by the method of boiling with KCN at acidic pH (Frenkel et al., 1980). After centrifuging, the supernatant is used for the assay. Then a paper chromatography of the extract is carried out. The filter paper is dried, cut into pieces, and used as samples for the microbiological assay. Samples usually need to be diluted for the microbiological method, and a B₁₂ assay medium is required. When *L. leishmannii* (ATCC 7830) is employed, the turbidity (%T) of the *L. leishmannii* test culture is measured at 600 nm. Microbiological methods are very sensitive, but they are usually tedious and time consuming, for it takes long to prepare and incubate the samples and the tissue must be cultured and the strain preserved. A well-trained full-time technician is also necessary. In addition, when *L. leichmanii* is used, vitamin B₁₂ analogs inactive for humans are also determined (Watanabe et al., 1998).

7.10 VITAMIN C

7.10.1 GENERALITIES

L-Ascorbic acid (AA) and L-dehydroascorbic acid (DHAA) are the two biologically vitamin C active compounds; AA is a strong reducing agent and DHAA is the

oxidized derivative of the AA. Interconversions of these compounds are carried out by glutathione dehydrogenase and ascorbate oxidase, among other enzymes. Vitamin C is a strong reducing agent and thus an important antioxidant. Vitamin C biochemical functions are based on its ability to donate one or two electrons.

Vitamin C is readily lost in cooking because of its water solubility. The availability of this vitamin is higher whenever vegetables are eaten raw, so fresh fruit and salad are the most reliable sources for vitamin C in the diet. Vitamin C can be depleted when fresh food is stored for a long time. Vitamin C can be oxidized to dehydroascorbate and then irreversibly degraded by hydrolytic opening of the lactone ring, which occurs in the presence of oxygen. Alkali, heat, light, and copper enhance the rate of oxidation.

Plant origin foods are the almost exclusive sources of vitamin C. In most developed countries this vitamin is used as a dietary supplement and chemical preservative, so soft drinks, cereals, cakes, confectionery, and fish and meat products might become an important dietary source of vitamin C. West Indian cherries, blackcurrants, oranges, lemons, strawberries, and kiwi fruit, among others, are good vitamin C sources, as well as most green leafy vegetables and also potatoes (the new ones in particular), because of the large amount generally eaten.

7.10.2 ANALYTICAL METHODS

Vitamin C analysis must be performed at low pH and, if necessary, in the presence of a chelating agent because this vitamin can be easily oxidized, especially at high pH conditions. The presence of Cu^{2+} and Fe^{3+} ions also contributes to this process. The biologically active DHAA can be reconverted to AA by reduction.

7.10.2.1 Physicochemical Determination

- Chemical methods: These are based on the power of L-AA as a reducing agent. These techniques are more rapid, more reproducible, and less specific than bioanalysis and microbiological assays for the complex biological relationship among the compounds that show vitamin C activity, as well as the similarities between these molecules and other that are not active. Chemical methods can be divided into two groups. The first one includes the determination of the reducing form of L-AA. The second one measures the total “vitamin C,” represented by L-AA, DHAA and 2,3-diketogulonic acid.

At present, for AA extraction, most methods use metaphosphoric acid. Sometimes, samples are cleaned up by solid-phase chromatography on a Sep-pak C₁₈ cartridge.

The first chemical methods were based on the oxidizing-reducing properties of L-AA or on its property of developing colored hydrazides by reaction with anyline diazotized derivatives. The extract is a reducing agent and can be measured by treatment with an oxidizing agent such as 2,6-dichlorophenolindophenol, proposed in 1927 by Tillmans. Other suitable agents are iodine and methylene-blue. About 50 years ago, “total”

- vitamin C was determined by reaction of 2,3-diketogulonic acid with 2,4-dinitrophenylhydrazine; after adding sulfuric acid, the oxazone formed gave a red color that could be read at 540 nm (Roe et al., 1948).
- **Titration:** The extract is dissolved with a mixture of metaphosphoric acid, glacial acetic acid, and water. After shaking and adding a starch solution, a titration with iodine solution is carried out.
 - **3,6-Dichlorophenolindo titrimetric method** (AOAC Int. 967.21, 45.1.14, 1995): L-AA is oxidized to L-DHAA by 2,6-dichloroindophenol. In the presence of significant amounts of iron or copper in the matrix to be analyzed, a chelating agent (such as ethylenediaminetetraacetic acid) should be included with the extraction. After the vitamin C extraction steps, sample extract is diluted. Three replicates of both standard and sample are titrated with 3,6-dichlorophenolindo solution. The red-color end point should last at least 10 sec to be valid. With colored samples, vitamin C needs to be determined by observing the change of transmittance at 545 nm. L-DHAA can be determined by first converting it to L-AA with a suitable reducing agent.
 - **Electrochemical methods:** These methods for determining L-AA are simpler. Polarography developed by Lindquist and Ferroha in 1975 is very specific and requires a low amount of sample and a minimum preparation.
 - **Enzymatic determination:** AA is transformed into DHAA using ascorbate oxidase.
 - **Colorimetric determination:** AA is oxidized with 2,6-dichlorophenolindophenol into DHAA and then it is converted into oxazone with 2,4-dinitrophenylhydrazine. After recoloring with sulfuric acid, it is finally measured at 520 nm.
 - **Microfluorometric method** (AOAC Int. 967.22, 45.1.15, 1995): A fluorescent quinoxaline compound is formed upon reaction of DHAA with *o*-phenylenediamine and measured at $\text{Ex} = 356 \text{ nm}$, $\text{Em} = 440 \text{ nm}$. With this method, it is possible to measure both AA and DHAA, for the former is previously oxidized to DHAA. To compensate for the presence of interfering substances, blanks need to be run using boric acid prior to the addition of the diamine solution. In blanks, development of fluorescent quinoxaline is prevented by the formation of H_3BO_3 -DHAA complex prior to addition of the phenylenediamine solution.
 - **HPLC determination:** Nowadays, HPLC separations are the most used because of the high sensitivity and selectivity of this technique (Rougereau et al., 1997; Cserháti and Forgács, 1999). For determining "total vitamin C," both AA and DHAA are determined. However, in some procedures, AA is separated. Vitamin C extraction is carried out using several chemicals (HPO_3 , TCA, EDTA, EDTA and citric acid, water, phosphate buffer), generally followed by dilution and injection of the samples.

Special preparation and extraction techniques have been described such as DHAA reduction, HPO_3 /ethanol utilization, enzymatic oxidation and *o*-phenylenediamine

derivatization, oxidation of AA followed by 2,4-dinitrophenylhydrazine (DNFH) derivatization, and HClO_4 , among many others.

Normal-phase adsorption HPLC has been employed, as well as ion-exchange chromatography, bounded- NH_2 partition chromatography, reversed-phase chromatography, and ion pair reversed-phase chromatography. C_{18} columns have been the most employed. RP-8, NH_2 , and silica columns have been also employed. UV detection has been the most used technique. However, electrochemical detection, as well as fluorescence after derivatization, can also be used.

When an NH_2 column is used, the retention time is strongly influenced by temperature (Gilpin and Sisco, 1980). A 35°C column temperature may be good for AA analysis, because a good retention time is obtained without affecting the stability of this vitamin. The problem of using reversed-phase HPLC on an NH_2 column is that after about 100 injections the column is worn out, probably due to the Schiff's base formation from NH_2 groups. However, both C_8 and C_{18} columns do not show this problem.

7.10.2.2 Microbiological Studies

For qualitative estimations, they are quicker but less used than chemical methods. *Myrothecium verrucacia* can be used (Meyer et al., 1965).

7.10.2.3 Biological Methods

In 1922 Sherman et al., described a bioanalysis assay based on determining the minimum amount of vitamin C necessary for protecting the Guinea pig from scurvy. Bioanalyses are expensive, less precise, and time consuming. They have the advantage of measuring all the compounds with vitamin C activity, and excluding those which do not show that activity. Nowadays, bioanalysis based on dental structure histological changes is the more specific method.

REFERENCES

GENERAL REFERENCES

- Association of Vitamin Chemists, Inc. *Methods of Vitamin Assay*, 3rd ed., Interscience Publishers, New York, 1966.
- Basu, T.K. and Dickerson, J.W.T., *Vitamins in Human Health & Disease*, CAB International, U.K., 1996.
- Christie, A.A. and Wiggins, R.A., Developments in vitamin analysis, in *Developments in Food Analysis Techniques*, Vol. I, King, R.D., Ed., Applied Science Publishers, London, 1978, pp. 1–42.
- Combs, G., Fundamental aspects in nutrition & health, in *The Vitamins*, Academic Press, New York, 1992.
- Cserháti, T. and Forgács, E., *Chromatography in Food Science & Technology*, Technomic Publishing Company, Lancaster, PA, 1999.

- Cuellar-Rodríguez, S., Vitaminas, in *Farmacología de las Enfermedades Endocrinas, Metabólicas e Inmunológicas*, PNFC, Consejo General de Colegios Oficiales de Farmacéuticos de España, Ed., Acción Médica, Madrid, Barcelona, Spain, 2000.
- Dolan, J.W., Improving an ion-pairing method, *LC-GC Int.*, October, 624–628, 1996.
- Duggan, D.E., Bowman, R.L., Brodie, B.B., and Udenfriend S., A spectrophotofluorometric study of compounds of biological interest, *Arch. Biochem. Biophys.*, 68, 1–14, 1957.
- Dyke, S.F., *The Chemistry of the Vitamins*, Interscience Publishers, New York, 1965, chap. 2.
- Food and Agriculture Organization (FAO), Requirements of Vitamin A, Iron, Folate, & Vitamin B₁₂. Report of a Joint FAO/WHO Expert Consultation, FAO Food & Nutrition Series No. 23, Food & Agriculture Organization, Rome, 1988, p. 107.
- Favell, D.J., Vitamin analysis. Microbiological methods, *J. Micronutr. Anal.*, 7, 237–246, 1990.
- Friedrich, W., *Handbuch der Vitamine*, Urban & Schwarzenberg, Munich, 1987.
- García Almansa, A., Vitaminas (I and II), *Nutr. Clín.*, 3, 1983.
- Gregory, J.F., Methods of vitamin assay for nutritional evaluation of food processing, *Food Technol.*, January, 75–80, 1983.
- Hashmi, M., *Assay of Vitamins in Pharmaceutical Preparations*, John Wiley & Sons, New York, 1973.
- Hedlund, B., Analysis of vitamins in food by HPLC, in *Recent Developments in Food Analysis*, Baltes W., Czedik-Eysenberg, P.B., and Pfannhauser, W., Eds., Verlag-Chemie, Weinheim, 1982, pp. 27–32.
- Horwitt, M.K., Liebert, E., Kreisler, O., and Wittman, P., Investigations of human requirements for B-complex vitamins, Bulletin of the National Research Council No. 116, National Academy of Sciences, Washington, D.C., 1948, p. 106.
- Hozova, B. and Sorman, L., *Combination of Irradiation & Thermal Processing Food Irradiation*, Elsevier Science Publishers, London, 1991, pp. 207–234.
- Hozova, B. and Takacsova, M., The influence of combined storage procedures of foods on B vitamins content demonstrated at the example of heat sterilisation and irradiation, *Die Nahrung*, 38(4), 345–351, 1993.
- Hyttén, F.E., Nutrition, in *Clinical Physiology in Obstetrics*, Hyttén, F. and Chamberlain, G., Eds., Blackwell, Oxford, 1980, pp. 163–192.
- IUPAC, Commission on biochemical nomenclature, *Biochemistry*, 147(1), 1970.
- Jansen, B.C.P., *The Vitamins*, 2nd ed., Vol. V, Sebrell, W.H. and Harris, R.S., Eds., Academic Press, New York, 1972, p. 99.
- Leach, D., Stadalius, M., Berus, J., and Snyder, L., Reversed-phase HPLC of basic samples, *LC-GC Int.*, 1(5), 22–30, 1988.
- Leenheer, A.P. and Lambert, W.E., *Modern Chromatographic Analysis of the Vitamins*, Marcel Dekker, New York, 1985.
- Lehninger, A.L., *Biochemistry*, Worth Publishers, New York, 1983.
- López, C. and Cos, A., Vitaminas, in *Manual de Alimentación y Nutrición para Educadores*, López, C. and Vázquez, C., Eds., Caja Madrid, Madrid, 1992.
- Lyon, J., *Le Grand Livre des Vitamines*, Martínez, R., Ed., Barcelona, 1987.
- Macrae, R., Nicolson, I.A., Richardson, D.P., and Scott, K.J., Chromatographic determination of vitamins in dairy products, RSC Proc. in *Challenges in Dairy Analytical Techniques*, Reading, MA, 1984, pp. 155–166.
- Merck and Co., Inc., *Merck Index*, 11th ed., Merck & Co., Inc., Whitehouse Station, NJ, 1989.
- Morawski, J., Analysis of dairy products by HPLC, *Food Technol.*, 38(4), 70–78, 1984.
- National Research Council (NRC), *Recommended Dietary Allowances*, 10th ed., National Academy Press, Washington, D.C., 1989.

- Ottaway, P., Vitamins as technical aids in food processing, *Food Technol. Int.*, 111–114, 1994.
- Parris, N.A., Non-aqueous reversed-phase liquid chromatography. A neglected approach to the analysis of low polarity samples, *J. Chromatogr.*, 157, 161–170, 1978.
- Passmore, R. and Eastwood, M.A., *Human Nutrition and Dietetics*, 8th ed., Davidson, S. and Passmore, R., Eds., Churchill Livingstone, New York, 1986.
- Rougereau, A., Person, O., and Rougereau, G., Determination of vitamins, in *Analysis of Food Constituents*, Multon, J.-L., Ed. Wiley-VCH, New York, 1997.
- Shah J., Riboflavin, in *Methods of Vitamin Assay*, 4th ed. Augustin, J., et al., Eds., John Wiley & Sons, New York, 1984.
- Shortt, C., Micronutrients and health, I: vitamins, *Trends Food Sci. Technol.*, July, 17–19, 1990.
- Snyder, L.R., Glajch, J.L., and Kirkland, J.J., *Practical HPLC Method Development*, John Wiley & Sons, New York, 1988.
- Steiner, F., *Applications of Narrow-Bore Columns in HPLC*, Hewlett-Packard GmbH, Germany, 1992.
- Steiner, I., Hruschka, G., Washüttl, J., and Kroyer, G., Selected B-vitamins in foodstuffs after conventional cooking and microwave treatment, *Ernährung/Nutrition*, 17(4), 221–225, 1993.
- Strohecker, R. and Henning, H.M., *Vitamin Assay-Tested Methods*, Translated by D.D. Libman, Verlag-Chemie, Weinheim, 1966.
- Thurnham, D.I., Bender, D.A., Scott, J., and Halsted, C.H., Water-soluble vitamins, in *Human Nutrition and Dietetics*, 10th ed., Garrow, J.S., James, W.P.T., and Ralph, A., Eds., Churchill Livingstone, London, 2000.
- Toma, R. and Tabekhia, M., High performance liquid chromatographic analysis of B-vitamins in rice and rice products, *J. Food Sci.*, 44(1), 263–268, 1979.
- Uherova, R., Fedorova, N., and Dubravicky, J., The study of vitamin interaction with the reactive constituents of smoking agent, *Die Nahrung*, 37(1), 85–87, 1993.
- Uherova, R., Hozova, B., and Smirnov, V., The effect of microwave heating on retention of some B vitamins, *Food Chem.*, 46, 293–295, 1993.
- Vandemark, F. and Schmidt, G., Determination of water soluble vitamins in pharmaceutical preparations using liquid chromatography, *J. Liq. Chromatogr.*, 4(7), 1157–1171, 1981.
- Vivanco, F., Palacios, J.M., y Garcia Almansa, A., *Alimentación y Nutrición*, Dirección General de Sanidad, Ed., Sección de Nutrición, Instituto de Investigaciones Clínicas y Médicas, Madrid, Spain, 1990.
- Weerdhof, T., Wiersum, M., and Reissenweber, H., Application of liquid chromatography in food analysis, *J. Chromatogr.*, 83, 455–460, 1972.
- Welt, B. and Tong, C., Effect of microwave power and electromagnetic energy, *J. Microwave Power Electromagn. Energy*, 28(4), 187–195, 1993.
- Wills, R., Shaw, C., and Day, W., Analysis of water soluble vitamins by high pressure liquid chromatography, *J. Chromatogr. Sci.*, 15(July), 262–266, 1977.
- Woidich, H., Pfannahuser, W., and Blaicher, G., cited in *Modern Chromatographic Analysis of the Vitamins* (Leenheer, A.P. and Lambert, W.E., 1985), *Lebensm. Chem. Gericht. Chem.*, 32, 74, 1978.
- Yost, R.W., Ettre, L.S., and Conlon, R.D., In *Practical Liquid Chromatography-An Introduction*, 1st ed., The Perkin-Elmer Corporation, Norwalk, CT, 1981.

MULTIVITAMIN METHOD REFERENCES

- Ang, C. and Moseley, F., Determination of thiamin and riboflavin in meat and meat products by high-pressure liquid chromatography, *J. Agric. Food Chem.*, 28(3), 483–486, 1980.

- Barna, E. and Dworschak, E., Determination of thiamine (vitamin B₁) and riboflavin (vitamin B₂) in meat and liver by high-performance liquid chromatography, *J. Chromatogr.*, A 668, 359–363, 1994.
- Barna, E., Comparison of data obtained by HPLC and microbiological determination of riboflavin in ready-to-eat foods, *Acta Aliment.*, 21(1), 3–9, 1991.
- Bognar, A., Bestimmung von Riboflavin und Thiamin in Lebensmitteln mit Hilfe der Hochdruck-Flüssigkeits-Chromatographie (HPLC), *Dtsch. Lebensm. Rundsch.*, 77(12), 431–436, 1981.
- Böttcher, B., Böttcher, D., and Kluthe, R., An HPLC method for the simultaneous determination of B₁, B₂ and B₆ vitamins in food, *Akt. Ernähr.*, 14, 117–118, 1989.
- Chase, G., Landen, W., and Soliman, A., Method modification for liquid chromatographic determination of thiamine, riboflavin, and pyridoxine in medical foods, *J. Assoc. Off. Anal. Chem. Int.*, 76(6), 1276–1280, 1993.
- Chase, G., Landen, W., Eitenmiller, R., and Soliman, A., Liquid chromatographic determination of thiamine, riboflavin, and pyridoxine in infant formula. *J. Assoc. Off. Anal. Chem. Int.*, 75(3), 561–565, 1992.
- Fellman, J., Artz, W., Tassinari, P., Cole, C., and Augustin, J., Simultaneous determination of thiamin and riboflavin in selected foods by high-performance liquid chromatography, *J. Food Sci.*, 47, 2048–2050, 1982.
- Fernando, S. and Murphy, P., HPLC determination of thiamin and riboflavin in soybeans and tofu, *J. Agric. Food Chem.*, 38, 163–167, 1990.
- Finglas, P. and Faulks, R., The HPLC analysis of thiamin and riboflavin in potatoes, *Food Chem.*, 15, 37–44, 1984.
- Gauch, R., Leuenberger, U., and Müller, U., Bestimmung der wasserlöslichen Vitamine B₁, B₂, B₆ und B₁₂ in Milch durch HPLC, *Z. Lebensm. Unters. Forsch.*, 195, 312–315, 1992.
- Hägg, M. and Kumpulainen, J., Thiamine and riboflavin contents in domestic and imported cereal products in Finland, *J. Food Comp. Anal.*, 6, 299–306, 1993.
- Hägg, M. and Kumpulainen, J., Thiamine and riboflavin contents in finnish pig, heifer, and cow livers and in pork loin, *J. Food Comp. Anal.*, 7, 1–6, 1994a.
- Hägg, M. and Kumpulainen, J., Thiamine and riboflavin contents of finnish breads and their corresponding flours, *J. Food Comp. Anal.*, 7, 94–101, 1994b.
- Hägg, M., Effect of various commercially available enzymes in the liquid chromatographic determination with external standardization of thiamine and riboflavin in foods, *J. Assoc. Off. Anal. Chem. Int.*, 77(3), 681–686, 1994.
- Hasselmann, C., Franck, D., Grimm, P., Diop, P., and Soules, C., High-performance liquid chromatographic analysis of thiamin and riboflavin in dietetic foods, *J. Micronutr. Anal.*, 5, 269–279, 1989.
- Kamman, J., Labuza, T., and Warthesen, J., Thiamin and riboflavin analysis by high-performance liquid chromatography, *J. Food Sci.*, 45, 1499–1504, 1980.
- Mauro, D. and Wetzel, D., Simultaneous determination of thiamine and riboflavin in enriched cereal based products by high-performance liquid chromatography using selective detection, *J. Chromatogr.*, 299, 281–287, 1984.
- Nail, P.A., Thomas, M.R., and Eakin, B.S., The effect of thiamin and riboflavin supplementation on the level of those vitamins in human breast milk and urine, *Am. J. Clin. Nutr.*, 33, 198–204, 1980.
- Reyes, E. and Subryan, L., An improved method of simultaneous HPLC assay of riboflavin and thiamin in selected cereal products, *J. Food Comp. Anal.*, 2, 41–47, 1989.
- Sims, A. and Shoemaker, D., Simultaneous liquid chromatographic determination of thiamine and riboflavin in selected foods, *J. Assoc. Off. Anal. Chem. Int.*, 76(5), 1156–1160, 1993.

- Skurray, G., A rapid method for selectively determining small amounts of niacin, riboflavin and thiamine in foods, *Food Chem.*, 7, 77–80, 1981.
- Wehling, R. and Wetzel, D., Simultaneous determination of pyridoxine, riboflavin, and thiamin in fortified cereal products by high-performance liquid chromatography, *J. Agric. Food Chem.*, 32, 1326–1331, 1984.
- Wimalasiri, P. and Wills, R., Simultaneous analysis of thiamin and riboflavin in foods by high-performance liquid chromatography, *J. Chromatogr.*, 318, 412–416, 1985.

SPECIFIC VITAMIN B₁ REFERENCES

- Abdel-Kader, Z., Comparison of AOAC and high-performance liquid chromatographic methods for thiamin determination in foods, *Food Chem.*, 43, 393–397, 1992.
- Association of Official Analytical Chemists (AOAC), Thiamine (vitamin B₁) in foods. Fluorometric method. Final action, No. 942.23, 15th ed., Arlington, VA, 1990.
- Ayi, B., Yuhas, D., Moffett, K., Joyce, D., and Deangelis, N., Liquid chromatographic determination of thiamine in infant formula products by using ultraviolet detection, *J. Assoc. Off. Anal. Chem.*, 68(6), 1087–1092, 1985.
- Bailey, A. and Finglas, P., A normal phase high performance liquid chromatographic method for the determination of thiamin in blood and tissue samples, *J. Micronutr. Anal.*, 7, 147–157, 1990.
- Bauer, A., Steger, U., and Wallnöfer, P., Thiamingehalt von Grünkern in Abhängigkeit von der Art der küchentechnischen Verarbeitung, *Dtsch. Lebensm.-Rundsch.*, 87(6), 180–182, 1991.
- Bettendorff, L., Grandfils, C., De Rycker, C., and Schoffeniels, E., Determination of thiamine and its phosphate esters in human blood serum at femtomole levels, *J. Chromatogr.*, 382, 297–302, 1986.
- Bognar, A., Determination of vitamin B₁ in food by high-performance liquid chromatography and postcolumn derivatization, *Fresenius J. Anal. Chem.*, 343, 155–156, 1992.
- Bötticher, B. and Bötticher, D., Simple rapid determination of thiamin by a HPLC method in foods, body fluids, urine and faeces, *Int. J. Vit. Nutr. Res.*, 56, 155–159, 1986.
- Ellefson, W., Thiamine, in *Methods of Vitamin Assay*, 4th ed., Augustin, J. et al., Eds., John Wiley & Sons, New York, 1984.
- Fox, J., Ackerman, S., and Thayer, D., Fluorometric determination of thiamine vitamers in chicken, *J. Assoc. Off. Anal. Chem. Int.*, 75(2), 346–354, 1992.
- Gubler, C., Thiamine, in *Handbook of Vitamins*, 2nd ed., Machlin, L.J., Ed., Marcel Dekker, New York, 1991.
- Herve, C., Beyne, P., and Delacoux, E., Determination of thiamine and its phosphate esters in human erythrocytes by high-performance liquid chromatography with isocratic elution, *J. Chromatogr.*, B, 653, 217–220, 1994.
- Hilker, D.M. and Clifford, A.J., Thiamin analysis and separation of thiamin phosphate esters by high-performance liquid chromatography, *J. Chromatogr. Biomed. Appl.*, 231, 433–438, 1982.
- Kawasaki, C., Modified thiamine compounds, in *Vitamins and Hormones*, Vol. XXI, Harris, R.S., Ed., Academic Press, New York, 1963.
- Laschi-loquerie, A., Vallas, S., Viollet, J., Leclercq, M., and Fayol, V., High performance liquid chromatographic determination of total thiamine in biological and food products, *Int. J. Vit. Nutr. Res.*, 62, 248–251, 1992.
- McCormick, D.B., Thiamin, in *Modern Nutrition in Health and Disease*, 7th ed., Shils, M.E. and Young, V.R., Eds., Lea & Febiger, Philadelphia, 1988, pp. 355–361.

- Nobile S., Savage V., and Huber U., A new procedure for the determination of thiamine in foods, *Int. J. Vit. Nutr. Res.*, 42, 444–450, 1972.
- Ollilainen, V., Vahteristo, L., Uusi-Raura, A., Varo, P., Koivistoinen, P., and Huttunen, J., The HPLC determination of total thiamin (Vitamin B₁) in foods, *J. Food Comp. Anal.*, 6, 152–165, 1993.
- Ryan, M. and Ingle, J., Fluorometric reaction rate method for the determination of thiamine, *Anal. Chem.*, 52, 2177–2184, 1980.
- Sander, S., Hahn, A., Stein, J., and Rehner, G., Comparative studies on the high-performance liquid chromatographic determination of thiamine and its phosphate esters with chloroethylthiamine as an internal standard using pre- and postcolumn derivatization procedures, *J. Chromatogr.*, 558, 115–124, 1991.
- Valls, F., Checa, M.A., Fernández Muiño, M.A., and Sancho, M.T., Determination of thiamin in cooked sausages, *J. Agric. Food Chem.*, 47, 170–173, 1999.
- Vanderslice, J. and Huang, M., Liquid chromatographic analysis of thiamin and its phosphates in food products using amprolium as an internal standard, *J. Micronutr. Anal.*, 2, 189–199, 1986.
- Vidal-Valverde, C. and Reche, A., An improved high performance liquid chromatographic method for thiamin analysis in foods, *Z. Lebensm. Unters. Forsch.*, 191, 313–318, 1990a.
- Wielders, J. and Mink, C., Quantitative analysis of total thiamine in human blood, milk and cerebrospinal fluid by reversed-phase ion-pair high-performance liquid chromatography, *J. Chromatogr.*, 277, 145–156, 1983.
- Williams, R.D., Mason, H.L., Smith, B.F., and Wilder, R.M., Induced thiamin deficiency and the thiamine requirement of man: further observations, *Arch. Int. Med.*, 69, 721–738, 1942.

SPECIFIC VITAMIN B₂ REFERENCES

- Association of Official Analytical Chemists (AOAC), Riboflavin (Vitamin B₂) in foods and vitamin preparations. Fluorometric Method. First action 1970. Final action 1971, No. 970.65, 15th ed., Arlington, VA, 1990.
- Ashoor, S., Knox, M., Olsen, J., and Deger, D., Improved liquid chromatographic determination of riboflavin in milk and dairy products, *J. Assoc. Off. Anal. Chem.*, 68(4), 693–696, 1985.
- Bilic, N. and Sieber, R., Determination of flavins in dairy products by high-performance liquid chromatography using sorboflavin as internal standard, *J. Chromatogr.*, 511, 359–366, 1990.
- Bourquin, A. and Sherman, H.C., Quantitative determination of vitamin B₂, *J. Am. Chem. Soc.*, 53, 3501, 1931.
- Cooperman, J.M. and López, R., Riboflavin, in *Handbook of Vitamins*, 2nd ed., Machlin, L.J., Ed., Marcel Dekker, New York, 1991.
- Johnsson, H. and Branzell, C., High performance liquid chromatographic determination of riboflavin in food. A comparison with a microbiological method, *Int. J. Vit. Nutr. Res.*, 57, 53–58, 1987.
- Kader, Z. and Ryley, J., The riboflavin content of blanched soya beans, a comparison of three methods of analysis, *J. Micronutr. Anal.*, 4, 169–175, 1988.
- Lumley, I.D. and Wiggins, R.A., Determination of riboflavin and flavin mononucleotide in foodstuffs using high-performance liquid chromatography and a column-enrichment technique, *Analyst*, 106(10), 1103–1108, 1981.

- Matts, F., Riboflavin, in *Vitamins in Medicine*, 4th ed. Vol. I. Barker, B. and Bender, D. Eds., William Heinemann Medical Books LTD., London, 1980.
- Ollilainen, V., Mattila, P., Varo, P., Koivistoinen, P., and Huttunen, J., The HPLC determination of total riboflavin in foods, *J. Micronutr. Anal.*, 8, 199–207, 1990.
- Reyes, E., Norris, K., Taylor, C., and Potts, D., Comparison of paired-ion liquid chromatographic method with AOAC fluorometric and microbiological method for riboflavin determination in selected foods, *J. Assoc. Off. Anal. Chem.*, 71(1), 16–19, 1988.
- Russell, L. and Vanderslice, J., Comments on the standard fluorometric determination of riboflavin in foods and biological tissues, *Food Chem.*, 43, 79–82, 1992a.
- Suhara, T., Torida, S., Ito, K., and Fukumoto, Y., Determination of vitamin B₂ in feed premixes by high-performance liquid chromatography, *Shiryo Kenkyu Hokoku (Tokyo Hishiryō Kensasho)*, 10, 152–163, 1985.
- Valls, F., Sancho, M.T., Fernández Muiño, M.A., and Checa, M.A., Determination of total riboflavin in cooked sausages, *J. Agric. Food Chem.*, 47, 1067–1070, 1999.
- Vidal-Valverde, C. and Reche, A., Reliable system for the analysis of riboflavin in foods by high performance liquid chromatography and UV detection, *J. Liq. Chromatogr.*, 13(10), 2089–2101, 1990b.
- Watada, A. and Tran, T., A sensitive high-performance liquid chromatography method for analyzing riboflavin in fresh fruits and vegetables, *J. Liq. Chromatogr.*, 8(9), 1651–1662, 1985.
- Woodcock, E.A., Warthesen, J.J., and Labuza, T.P., Riboflavin photochemical degradation in pasta measured by high-performance liquid chromatography, *J. Food Sci.*, 47, 545–549, 1982.

SPECIFIC VITAMIN B₃ REFERENCES

- Balschukat, D. and Kress, E., Use of column switching for the determination of niacinamide in compound feed, *J. Chromatogr.*, 502, 79–85, 1990.
- Cantoni, C., Cattaneo, P., and Lauri, S., Sulla nicotinamide nei prodotti carni e dati sperimentali, *Ing. Alimentare*, 5, 42–43, 1992.
- Dawson, K., Unklesbay, N., and Hedrick, H., HPLC determination of riboflavin, niacin, and thiamin in beef, pork, and lamb after alternate heat-processing methods, *J. Agric. Food Chem.*, 36, 1176–1179, 1988.
- Horwitt, M.K., Harper, A.E., and Henderson, L.M., Niacin-tryptophan relationships for evaluating niacin equivalents, *Am. J. Clin. Nutr.*, 34, 423–427, 1981.
- Fontaine, J. and Hörr, J., Determination of supplemented nicotinic acid or nicotinamide in compound feed by HPLC after cleaning of the feed extract with cationic sorbent extraction, *Agribiol. Res.*, 46(1), 10–19, 1993.
- Fujita, T., Kanbe, K., and Sasaki, K., Studies on high-performance liquid chromatographic determination of nicotinic acid in foods, *Seikatsu Eisei*, 27, 30, 1983.
- Gigliotti, C. and Daghetta, A., Indagine conoscitiva sul contenuto di acido nicotinico negli insaccati freschi, *Ind. Alimen.*, 32 (February), 113–120, 1993.
- Hamano, T., Mitsuhashi, Y., Aoki, N., and Yamamoto, S., Simultaneous determination of niacin and niacinamide in meats by high-performance liquid chromatography, *J. Chromatogr.*, 457, 403–408, 1988.
- Johnson, B.C., The microbiological determination of nicotinic acid, nicotinamide, and nicotinic acid, *J. Biol. Chem.*, 159, 227, 1945.
- Kitada, Y., Inone, M., Tamase, K., Imou, M., Hasuike, A., Sasaki, M., and Tanigawa, K., Analysis of nicotinic acid and nicotinamide in foods by ion-pair HPLC, *Eiyo Shokuryo*, 35, 121–124, 1982.

- McKee, R.N., Kang-Lee, Y.A., Panaqua, M., and Swendseid, M.E., Determination of nicotinamide and metabolic products in urine by high performance liquid chromatography, *J. Chromatogr.*, 230, 309–317, 1982.
- Sakai, H., Suzuki, A., Izawa, K., and Niimi, T., Determination of nicotinamide in premixes by high performance liquid chromatography, *Shiryo Kenkyu Hokoku (Tokyo Hishiryo Kensasho)*, 10, 122–129, 1985.
- Snell, E.E. and Wright, L.D., A microbiological method for the determination of nicotinic acid, *J. Biol. Chem.*, 139, 675, 1941.
- Takatsuki, K., Suzuki, S., Sato, M., Sakai, K., and Ushizawa, I., Liquid chromatographic determination of free and added niacin and niacinamide in beef and pork, *J. Assoc. Off. Anal. Chem.*, 70(4), 698–702, 1987.
- Trugo, L., Macrae, R., and Trugo, N., Determination of nicotinic acid in instant coffee using high-performance liquid chromatography, *J. Micronutr. Anal.*, 1, 55–63, 1985.
- Tyler, T. and Genzale, J., Liquid chromatographic determination of total niacin in beef, semolina, and cottage cheese, *J. Assoc. Off. Anal. Chem.*, 73(3), 467–469, 1990.
- Valls, F., Sancho, M.T., Fernández Muiño, M.A., and Checa, M.A., Simultaneous determination of nicotinic acid and nicotinamide in cooked sausages, *J. Agric. Food Chem.* 48, 3392–3395, 2000.
- Vidal-Valverde, C. and Reche, A., Determination of available niacin in legumes and meat by high-performance liquid chromatography, *J. Agric. Food Chem.*, 39, 116–121, 1991.
- Williams, W.L., Yeast microbiological method for determination of nicotinic acid, *J. Biol. Chem.*, 166, 397, 1946.
- Yoshida, K., Yamamoto, Y., and Fujiware, M., Simple analytical method for niacin (nicotinic acid) and nicotinamide in foods by high-performance liquid chromatography, *Shokuhin Eiseigaku Zasshi*, 23(6), 428–433, 1982.

SPECIFIC VITAMIN B₆ REFERENCES

- Ang, C., Cenciarelli, M., and Eitenmiller, R., A simple liquid chromatographic method for determination of B₆ vitamers in raw and cooked chicken, *J. Food Sci.*, 53(2), 371–375, 1988.
- Bessey, O.A., Adam, D.J., and Hansen, A.E., Intake of vitamin B₆ and infantile convulsions: a first approximation of requirements of pyridoxine in infants, *Pediatrics*, 20, 33–44, 1957.
- Bitsch, R. and Möller, J., Evaluation of the vitamin B₆ content in foods by HPLC analysis, *Dtsch. Forschungsgem.*, grant Bi 218/5-1, 77–79, 1988.
- Bitsch, R., and Möller, J., Analysis of B₆ vitamers in foods using a modified high-performance liquid chromatographic method, *J. Chromatogr.*, 463, 207–211, 1989.
- Bognar, A., Bestimmung von Vitamin B₆ in Lebensmitteln mit Hilfe der HochdruckFlüssig-Chromatographie (HPLC), *Z. Lebensm. Unters. Forsch.*, 181, 200–205, 1985.
- Bognar, A., Studies on the influence of cooking on the vitamin B₆ content of food, *Bioavailability*, 93, 346–351, 1993.
- Coburn, S.P. and Mahuren, J.D., A versatile cation-exchange procedure for measuring the seven major forms of vitamin B₆ in biological samples, *Anal. Biochem.*, 129, 310–317, 1983.
- Driskell, J.A., Vitamin B₆, in *Handbook of Vitamins-Nutritional, Biochemical and Clinical Aspects*, Machlin, L.J., Ed., Marcel Dekker, New York, 1984, pp. 379–401.
- Gregory, J.F. and Kirk, J.R., cited in *Determination of Vitamin B₆ in Animal Tissues by Reverse-Phase High-Performance Liquid Chromatography* (Gregory, J.F., Manley, D.B., and Kirk, J.R., 1981), *Am. J. Clin. Nutr.*, 32, 879, 1979.

- Gregory, J.F. and Sartain, D.B., Improved chromatographic determination of free and glycosylated forms of vitamin B₆ in foods, *J. Agric. Food Chem.*, 39, 899–905, 1991.
- Gregory, J.F., Methods for determination of vitamin B₆ in foods and other biological materials: a critical review, *J. Food Comp. Anal.*, 1, 105–123, 1988.
- Gregory, J.F., Manley, D.B., and Kirk, J.R., Determination of vitamin B₆ in animal tissues by reverse-phase high-performance liquid chromatography, *J. Agric. Food Chem.*, 29, 921–927, 1981.
- Hozova, B., Sorman L., Dudasova S., and Rajniakova A., Study of retention of vitamin B₆ in non-traditionally canned foods on using an experimental plan method, *Die Nahrung*, 32(4), 413–414, 1988.
- Lim, K., Young, R., and Driskell, J., Separation of vitamin B₆ components by high-performance liquid chromatography, *J. Chromatogr.*, 188, 285–288, 1980.
- Möller, J. and Bitsch, R., Einsatz der Hochleistungs-flüssigchromatographie (HPLC) zur simultanen Bestimmung der Pyridoxin-derivate aus Biologischen Materialien, *Ernährung. Umsch.*, 35(9), 324–328, 1988.
- Reitzer-Bergaentzle, M., Marchioni, E., and Hasselmann, C., HPLC determination of vitamin B₆ in foods after precolumn derivatization of free and phosphorylated vitamers into pyridoxol, *Food Chem.*, 48, 321–324, 1993.
- Schoonhoven, J., Schrijver, J., Berg, H., and Haenen, G., Reliable and sensitive high-performance liquid chromatographic method with fluorometric detection for the analysis of vitamin B₆ in foods and feeds, *J. Agric. Food Chem.*, 42, 1475–1480, 1994.
- Valls, F., Sancho, M.T., Fernández Muiño, M.A., and Checa, M.A., Determination of vitamin B₆ in cooked sausages, *J. Agric. Food Chem.*, 49, 38–41, 2001.
- Vanderslice, J., Maire, C., Doherty, R., and Beecher, G., Sulfosalicylic acid as an extraction agent for vitamin B₆ in food, *J. Agric. Food Chem.*, 28, 1145–1149, 1980.
- Vanderslice, J., Brownlee, S., and Cortissoz, M., Liquid chromatographic determination of vitamin B₆ in foods, *J. Assoc. Off. Anal. Chem.*, 67(5), 999–1007, 1984.
- Vanderslice, J.T., Stewart, K.K., and Yarnas, M.M., Liquid chromatographic separation and quantification of B₆ vitamers and their metabolite, pyridoxic acid, *J. Chromatogr.*, 176, 280–285, 1979.
- Williams, A.K. and Cole, P.D., Vitamin B₆: ion exchange chromatography of pyridoxal, pyridoxol and pyridoxamine, *J. Agric. Food Chem.*, 23, 915–916, 1975.
- Wong, F.F., Analysis of vitamin B₆ in extractives of food materials by high-performance liquid chromatography, *J. Agric. Food Chem.*, 26, 1444–1446, 1978.
- Yasumoto, K., Tadera, K., Tsuji, H., and Mitsuda, H., Semi-automated system for analysis of vitamin B₆ complex by ion-exchange column chromatography, *J. Nutr. Sci. Vitaminol.*, 21, 117–127, 1975.

SPECIFIC BIOTIN REFERENCES

- Desvene, P.L., Coustal, S., and Frappier, F., Separation of biotin and its analogs by high-performance liquid chromatography: convenient labeling for ultraviolet or fluorimetric detection, *Anal Biochem.*, 128, 359–362, 1983.
- Grosvenor, A.L., Feltus, A., Conover, R.C., Daunert, S., and Anderson, K.W., Development of binding assays in microfabricated picoliter vials: an assay for biotin, *Anal Chem.*, 72, 2590–2594, 2000.
- Hayakawa, K. and Oizumi, J., Determination of free biotin in plasma by liquid chromatography with fluorimetric detection, *J. Chromatogr.*, 413, 247–250, 1987.
- Hentz, N.G. and Bachas, L.G., Class-selective detection systems for liquid chromatography-based on the streptavidin-biotin interaction, *Anal. Chem.*, 67, 1014–1018, 1993.

- Przyjazny, A., Hentz, N.G., and Bachas, L.G., Sensitive and selective liquid chromatographic postcolumn reaction detection system for biotin and biocytin using a homogeneous fluorophore linked assay, *J. Chromatogr.*, 654, 79–86, 1993.
- Lahély, S., Ndaw, S., Arella, F., and Hasselmann, C., Determination of biotin in foods by high-performance liquid chromatography with postcolumn derivatization and fluorimetric detection, *Food Chem.*, 65, 253–258, 1999.
- Livaniou, E., Costopoulou, D., Vassiliadou, I., Leondiadis, L., Nyalala, J.O., Ithakissios, D.S., and Evangelatos, G.P., Analytical techniques for determining biotin, *J. Chromatogr.*, A, 881, 331–343, 2000.
- Stein, J., Hahn, A., Lembke, B., and Rehner, G., High-performance liquid chromatographic determination of biotin in biological materials after crown ether-catalized fluorescence derivatization with panacyl bromide, *Anal. Biochem.*, 200, 89–94, 1992.
- Whitehead, C.C., Armstrong, J.A., and Waddington, D., The determination of the availability to chicks of biotin in feed ingredients by a bioassay based on the response of blood pyruvate carboxylase (EC 6.4.1.1.) activity, *Br. J. Nutr.*, 48, 81–88, 1982.
- Yoshida, T., Uetake, A., Nakai, C., Nimura, M., and Kinoshita, T., Liquid chromatographic determination of biotin using 1-pyrenyldiazomethane as a precolumn fluorescent labeling reagent, *J. Chromatogr.*, 456, 421–426, 1988.

SPECIFIC FOLATE REFERENCES

- Day, B.P.F. and Gregory, J.F., III, Determination of folacin derivatives in selected foods by high performance liquid chromatography, *J. Agric. Food. Chem.*, 29, 374–377, 1981.
- Desouza, S. and Eitenmiller, R., Effects of different enzyme treatments on extraction of total folate from various foods prior to microbiological assay and radioassay, *J. Micronutr. Anal.*, 7, 37–57, 1990.
- Finglas, P.M., Faure, U., and Southgate, D.A.T., First BRC intercomparison on the determination of folates in food, *Food Chem.*, 46, 199–213, 1993.
- Finglas, P.M., Wigertz, K., Vahteristo, L., Witthöft, C., Southon, S., and De Froidmont-Görtz, I., Standardisation of HPLC techniques for the determination of naturally-occurring folates in food, *Food Chem.*, 64, 245–255, 1999.
- Flynn, L.M., Microbiological assays of folic acid activity in foods: changes in results with variation in experimental conditions and methods, *J. Assoc. Offic. Anal. Chem.*, 48, 1230–1235, 1965.
- Gregory, J.F., III, Sartain, D.B., and Day, B.P.F., Fluorometric determination of folacin in biological materials using high performance liquid chromatography, *J. Nutr.*, 114, 341–353, 1984.
- Holt, D.L., Wehling, R.L., and Zeece, M.G., Determination of native folates in milk and other dairy products by high-performance liquid chromatography, *J. Chromatogr.*, 449, 271–279, 1988.
- Luccock, M.D., Green, M., Priestnall, M., Daskalakis, I., Levene, M.I., and Hartley, R., Optimisation of chromatographic conditions for the determination of folates in foods and biological tissues for nutritional and clinical work, *Food Chem.*, 53, 329–338, 1995.
- Martin, J.I., Landen, W.I., Jr., and Soliman, A.G.M., Application of a trienzyme extraction for total folate determination in foods, *J. Assoc. Off. Anal. Chem.*, 73(5), 805–808, 1990.
- Pfeiffer, C.M., Rogers, L.M., and Gregory, J.F., Determination of folate in cereal grain food products using trienzyme extraction and combined affinity and reverse-phase liquid chromatography, *J. Agric. Food Chem.*, 45, 407–413, 1997.
- Phillips, D.R. and Wright, A.J.A., Studies on the response of *Lactobacillus casei* to different folate monoglutamates, *Br. J. Nutr.*, 47, 183–189, 1982.

- Rader, J.I., Weaver, C.M., and Angyal, G., Use of a microbiological assay with tri-enzyme extraction for measurement of pre-fortification levels of folates in enriched cereal-grain products, *Food Chem.*, 62(4), 451–465, 1998.
- Ridascreen®-Folsäure, Direct competitive ELISA enzymoinmunoassay for folic acid (Ref. 16311), r-biopharm, ATOM, Digen Ltd., U.K., 1998.
- Reingold, R.N., Perkins, E.G., and Picciano, M.F., Separation of folate derivatives by *in situ* paired-ion high pressure liquid chromatography, *J. Chromatogr.*, 190, 237–240, 1980.
- Tamura, T., Mizuno, Y., Johnston, K.E., and Jacob R.A., Food folate assay with protease, α -amilase and folate conjugase treatments, *J. Agric. Food Chem.*, 45, 135–139, 1997.
- Vatehristo, L., Ollilainen, V., Koivistoinen, P.E., and Varo, P., Improvements in the analysis of reduced folate monoglutamates and folic acid in food by high-performance liquid chromatography, *J. Agric. Food Chem.*, 44, 477–482, 1996a.
- Vatehristo, L., Finglas, P.M., Witthöft, C.M., Wigertz, K., Seale, R., and de Froidmont-Görtz, I., Third EU-MAT intercomparison study on food folates analysis using HPLC procedures, *Food Chem.*, 57(1), 109–111, 1996b.
- Wigertz, K. and Jägerstad, M., Comparison of a HPLC and radioprotein-binding assay for the determination of folates in milk samples, *Food Chem.*, 54(4), 429–436, 1995.

SPECIFIC VITAMIN B₁₂ REFERENCES

- Bennink, M.R. and Ono, K., Vitamin B₁₂, E and D contents of raw and cooked beef, *J. Food Sci.*, 47, 1786–1792, 1982.
- Casey, P.J., Speckman, K.R., Ebert, F.J., and Hobbs, W.E., Radioisotope dilution technique for determination of vitamin B₁₂ in foods, *J. Assoc. Off. Anal. Chem.*, 65, 85–88, 1982.
- Eitenmiller, R.R., Landen, W.O., and Augustin, J., Jr., Vitamin analysis, in *Food Analysis*, 2nd ed., Nielsen, S.S., Ed., An Aspen Publication, Chapman & Hall Food Science Titles, Gaithersburg, MD, 1998.
- Fie, M., Zee, J.A., and Amiot, J., Séparation et quantification des isomères de la vitamine B₁₂ dans le lait et certains produits laitiers par chromatographie liquide haute performance et par radio-essai, *Sci. Alim.*, 14, 763–775, 1994.
- Ford, S., Gallery, J., Nichols, A., and Shambee, M., High-performance liquid chromatographic analysis of the (cyanoquo) stereoisomers of several putative vitamin B₁₂ precursors, *J. Chromatogr.*, 537, 235–247, 1991.
- Frenkel, E.P., Prough, R., and Kitchens, R.L., Measurement of tissue vitamin B₁₂ by radioisotopic competitive inhibition assay and quantitation of tissue cobalamin fractions, *Methods Enzymol.*, 67, 31–40, 1980.
- Iwase, H. and Ono, I., Determination of cyanocobalamin in foods by high-performance liquid chromatography with visible detection after solid-phase extraction and membrane filtration for the precolumn separation of lipophilic species, *J. Chromatogr. A*, 771, 127–134, 1997.
- Jacobsen, D.W., Green, R., Quadros, E.V., and Montejano, Y.D., Rapid analysis of cobalamin coenzymes and related corrinoid analogues by high-performance liquid chromatography, *Anal. Biochem.*, 120, 394–403, 1982.
- Kralova, B., Rauch, P., and Cerna, J., Use of vitamin B₁₂ radioassay in the analysis of biological materials, mainly foods, *Nahrung*, 26, 803–810, 1982.
- Richardson, R.J., Favell, D.J., Gidley, G.C., and Jones, G.H., Application of a commercial radioassay test kit to the determination of vitamin B₁₂ in food, *Analyst*, 103, 865–868, 1978.
- Ridascreen®-Test, Direct competitive ELISA enzymoinmunoassay for vitamin B₁₂ (Ref. 16309), r-biopharm, ATOM, Digen Ltd., U.K., 1998.

- Schneider, Z., Purification and estimation of vitamin B₁₂, in *Comprehensive B₁₂*, Schneider, Z. and Stroinski, A., Eds., De Gruyter, Berlin, 1987, pp. 111–155.
- Watanabe, F., Takenaka, S., Abe, K., Tamura, Y., and Nakano, Y., Comparison of a microbiological assay and a fully automated chemiluminescent system for the determination of vitamin B₁₂ in food, *J. Agric. Food Chem.*, 46(4), 1433–1436, 1998.

SPECIFIC VITAMIN C REFERENCES

- Association of Official Analytical Chemists International., *Official Methods of Analysis*, 16th ed., AOAC International, Gaithersburg, MD, 1995.
- Barja De Quiroga, G., López, M., Pérez, R., and Rojas, C., Simultaneous determination of two antioxidants, uric and ascorbic acid, in animal tissue by high-performance liquid chromatography, *Anal. Biochem.*, 199, 81–85, 1991.
- Bessey, O.A. and King, C.G., The distribution of vitamin C in plant and animal tissues and its determination, *J. Biol. Chem.*, 103, 687, 1933.
- Buslig, B.S., Wilson, C.W., III, and Shaw, P.E., High-performance liquid chromatographic separation of carboxylic acids with anion-exchange and reverse-phase columns, *J. Agric. Food Chem.*, 30, 342–345, 1982.
- Carr, R.S., Bally, M.B., Thomas, P., and Neff, J.M., Comparison of methods for determination of ascorbic acid in animal tissues, *Anal. Chem.*, 55, 1229–1232, 1983.
- Coustard, J.M. and Sudraud, G., Séparation des acides ascorbique et isoascorbique par chromatographie de paires d'ions sur phase inverse, *J. Chromatogr.*, 219, 338–342, 1981.
- Dennison, D., Brawley, T., and Hunter, G., Rapid high-performance liquid chromatographic determination of ascorbic acid and combined ascorbic acid-dehydroascorbic acid in beverages, *J. Agric. Food Chem.*, 29, 927–929, 1981.
- Finley, J.M. and Duang, D., Resolution of ascorbic, dehydroascorbic and diketogulonic acids by paired-ion reversed-phase chromatography, *J. Chromatogr.*, 207, 449–453, 1981.
- Gilpin, R.K. and Sisco, W.R., cited in *Modern Chromatographic Analysis of the Vitamins* (Leenheer, A.P. and Lambert, W.E., 1985), *J. Chromatogr.*, 194, 285, 1980.
- Harris, L.J., Mapson, L.W., and Wang, Y.L., Vitamin methods. A simple potentiometric method for determining ascorbic acid suitable for use with colored extracts, *Biochem. J.*, 36, 183, 1942.
- Hedlund, B., Analysis of vitamins in food by HPLC, in *Recent Developments in Food Analysis*, Baltes, W., Czedik-Eysenberg, P.B., and Pfannhauser, W., Eds., Verlag Chemie, Weinheim, 1982, pp. 27–32.
- Iwase, H. and Ono, I., Determination of ascorbic acid and dehydroascorbic acid in juices by high-performance liquid chromatography with electrochemical detection using L-cysteine as precolumn reductant, *J. Chromatogr.*, A 654, 215–220, 1993.
- Kodaka, K., Inagaki, S., Ujie, T., Ueno, T., and Suda H., Determination of total vitamin C in foods by high-performance liquid chromatography, *Vitamin*, 59(5), 451–455, 1985.
- Kutnink, M. and Omaye, S., Determination of ascorbic acid, erythorbic acid, and uric acid in cured meats by high performance liquid chromatography, *J. Food Sci.*, 52(1), 53–56, 1987.
- Lindquist, J. and Ferroha, S.M., cited in *Modern Chromatographic Analysis of the Vitamins* (Leenheer, A.P. and Lambert, W.E., 1985), *Analyst (London)*, 100, 377, 1975.
- Meyer, B.J., Lagally, H., and Dodds, M.L., cited in *Modern Chromatographic Analysis of the Vitamins* (Leenheer, A.P. and Lambert, W.E., 1985), *Anal. Biochem.*, 4, 88, 1965.
- Moledina, K.H. and Flink, J.M., Determination of ascorbic acid in plant food products by high performance liquid chromatography, *Lebensm. Wiss. Unters. Technol.*, 15, 351–358, 1982.

- Parolari, G., L'acido ascorbico nelle carni, *Ind. Conserve*, 57, 19–21, 1982.
- Pelletier, O., Vitamin C (L-ascorbic and dehydro-L-ascorbic acid), in *Methods of Vitamin Assay*, 4th ed., Augustin, J., Klein, B.P., Becker, D.A., and Venugopal, P.B., Eds., John Wiley & Sons, New York, 1985, pp. 334–336, 338, and 341.
- Roe, J.H., Mills, M.B., Oesterling, M.J., and Dameron, C.M., cited in *Modern Chromatographic Analysis of the Vitamins* (Leenheer, A.P. and Lambert, W.E., 1985), *J. Biol. Chem.*, 174, 201, 1948.
- Rizzolo, A., Forni, E., and Polesello, A., HPLC assay of ascorbic acid in fresh and processed fruit and vegetables, *Food Chem.*, 14, 189–199, 1984.
- Sawamura, M., Ooishi, S., and Li, Z., Reduction of dehydroascorbic acid by sodium hydrosulfide and liquid chromatographic determination of vitamin C in citrus juices, *J. Sci. Food Agric.*, 53, 279–281, 1990.
- Schmall, M., Pifer, C.W., and Wollish, E.G., Determination of ascorbic acid by a new colorimetric reaction, *Anal. Chem.*, 25, 1486, 1953.
- Sherman, H.C., Lamer, V.K., and Campbell, H.L., The quantitative determination of the antiscorbic vitamin, *J. Am. Chem. Soc.*, 44, 165, 1922.
- Schüep, W. and Keck, E., Measurement of ascorbic acid and erythorbic acid in processed meat by HPLC, *Z. Lebensm. Unters. Forsch.*, 191, 290–292, 1990.
- Sood, S., Sartori, L., Wittmer, D., and Haney, W., High-pressure liquid chromatographic determination of ascorbic acid in selected foods and multivitamin products, *Anal. Chem.*, 48(6), 796–798, 1976.
- Speek, A., Schrijver, J., and Schreurs, W., Fluorometric determination of total vitamin C and total isovitamin C in foodstuffs and beverages by high-performance liquid chromatography with precolumn derivatization, *J. Agric. Food Chem.*, 32, 352–355, 1984.
- Tillmans, J., cited in *Modern Chromatographic Analysis of the Vitamins* (Leenheer, A.P. and Lambert, W.E., 1985), *Z. Lebensm. Unters. Forsch.*, 54, 33, 1927.
- Tsao, C.S. and Salimi, S.L., Differential determination of L-ascorbic acid and D-isoascorbic acid by reversed-phase high-performance liquid chromatography with electrochemical detection, *J. Chromatogr.*, 245(3), 355–358, 1982.
- Valls-Garcia, F., Determinación de vitaminas en productos cárnicos cocidos mediante cromatografía líquida de alta eficacia. I. Optimización de los métodos de extracción y analíticos. II. Evaluación de las pérdidas vitamínicas durante el proceso industrial de fabricación, Tesis Doctoral, Universidad de Burgos, 1997.
- Vanderslice, J. and Higgs, D., Chromatographic separation of ascorbic acid, isoascorbic acid, dehydroascorbic acid and dehydroisoascorbic acid and their quantitation in food products, *J. Micronutr. Anal.*, 4, 109–118, 1988.
- Vanderslice, J. and Higgs, D., Separation of ascorbic acid, isoascorbic acid, dehydroascorbic acid and dehydroisoascorbic acid in food and animal tissue, *J. Micronutr. Anal.*, 7, 67–70, 1990.

8 Carbohydrates and Other Electrochemically Active Compounds

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The development of analyses for foods and agricultural samples presents a major challenge to analytical chemistry, especially in regard to the determination of compounds that have poor optical detection properties (e.g., carbohydrates). Pulsed electrochemical detection (PED) is a revolutionary approach to the direct detection of numerous polar aliphatic compounds. This technique exploits the electrocatalytic activity of noble metal electrode surfaces to oxidize various polar functional groups. Electrode activity is maintained by the application of potential-time waveforms, which combines amperometric detection with on-line cleaning and reactivation. The full potential of PED is best realized when combined with high-performance liquid chromatography (HPLC). This chapter reviews the fundamental aspects of PED and summarizes food- and agricultural-related applications from simple carbohydrates to biogenic amines and thiocompounds.

8.1 INTRODUCTION

The development of HPLC methods for food and agricultural products is complicated by the poor optical detection properties of many of their major components (e.g., carbohydrates, oligosaccharides, polysaccharides). Although significant advances have occurred in the use of preinjection and postcolumn chemical derivatization to produce photometrically and electrochemically active adducts, the simplicity of sensitive direct detection in HPLC will always be preferred whenever available.

Electrochemical detection (ED) in HPLC has proven to be a powerful analytical technique for the determination of compounds containing electroactive groups. The high sensitivity and selectivity of ED is ideally suited for complex samples, as evinced by its application to the determination of neurotransmitters in complex biological samples (i.e., brain extracts). Neurotransmitters are typically aromatic compounds (e.g., phenols, aminophenols, catecholamines, and other metabolic amines), which are detected easily by anodic reactions at a constant (dc, detections at constant) applied potential at inert electrodes (Adams, 1969; Kissinger, 1984). The most common electrode materials are Au, Pt, and carbon. Electronic resonance in aromatic molecules stabilizes free-radical intermediate products of anodic oxidations, and, as a consequence, the activation barrier for the electrochemical reaction

is lowered significantly. In contrast, absence of π -resonance for aliphatic compounds results in very low oxidation rates, even though the reactions may be favored thermodynamically (Adams, 1969). Stabilization of free radical products from aliphatic compounds can be achieved alternatively via their adsorption to the surface of noble metal electrodes. Unfortunately, adsorption of organic molecules and free-radicals also has the consequence of fouling of the electrode and loss of its activity (Breiter, 1963; Gilman, 1967; Giner, 1964). The historical perspective of nonreactivity for aliphatic compounds at noble metal electrodes can be attributed to surface fouling as a result of high, but transient, catalytic activity.

Even for reversible redox couples that are considered to be well behaved, dc amperometry is often accompanied by the practice of disassembling the electrochemical cell and mechanically polishing the working electrode. In this manner, fouling from nonspecific adsorption processes and/or mechanistic consequences is physically removed from the electrode surface. An alternate approach is to combine ED with "on-line" cleaning. Hence, in order to maintain uniform and reproducible electrode activity at noble metal electrodes for polar aliphatic compounds, PED was developed (Hughes, Meschi, and Johnson, 1981).

8.2 HISTORICAL PERSPECTIVE

Virtually every publication concerning voltammetric data obtained on noble metal electrodes describes a procedure for electrode pretreatment to clean and reactivate electrodes that have become fouled by adsorption of solution impurities. Preparation and reactivation of noble metal electrodes are commonly achieved by the repetitive application of cyclic potential scans or alternated positive and negative potential pulses. In fact, the use of alternating positive and negative potential pulses to reactivate noble metal electrodes has been employed since the early 1900s (Armstrong, Himsworth, and Butler, 1934; Hammett, 1924). In the 1950s and 1960s, the development of hydrocarbon fuel cells inspired research on pulsed waveforms to maintain the activity of their noble metal electrodes (Breiter, 1963; Gilman, 1963, 1967; Giner, 1964). In the 1970s, the advent of ED in flow-injection and liquid chromatographic systems intensified the interest in pulsed waveforms to facilitate electroanalytical detection at noble metal electrodes (Clark, Fleishman, and Fletcher, 1972; MacDonald and Duke, 1973; Stulik and Hora, 1976).

Pulsed amperometric detection (PAD) was first introduced in 1981 for the detection of aliphatic alcohols at Pt electrodes (Hughes and Johnson, 1981; Hughes, Meschi, and Johnson, 1981). The Johnson group at Iowa State University pioneered research on pulsed waveforms at noble metal electrodes for the detection of polar aliphatic compounds. In conjunction with Dionex Corporation (Sunnyvale, CA), the first commercial detector dedicated to PAD was joined with high-performance anion-exchange chromatography (HPAEC) for the direct determination of carbohydrates (Edwards and Haak, 1983; Rocklin and Pohl, 1983). The application of triple-step potential-time waveforms in ED at noble metal electrodes was known as PAD (Edwards and Haak, 1983; Hughes and Johnson, 1982, 1983; Johnson and LaCourse, 1990; Rocklin and Pohl, 1983). PAD was soon followed by pulsed coulometric detection (PCD) (Neuberger and Johnson, 1988, 1990), which integrated the amperometric signal. Regardless of the specific

form of signal measurement, all of these techniques are now grouped under the title of PAD. Potential sweep-pulsed coulometric detection (PS-PCD) (Neuberger and Johnson, 1988; Welch et al., 1989), which incorporates a triangular potential scan to the detection step of PCD, has become known as integrated pulsed amperometric detection (IPAD) (LaCourse and Johnson, 1990) and less commonly as integrated voltammetric detection (IVD) (LaCourse, 1997). The “integrated” description refers to the coulometric rejection of the oxide background. All detection strategies based on the application of multistep potential-time waveforms at noble metal electrodes for ED in HPLC fall under the term PED (Johnson and LaCourse, 1990, 1992; LaCourse, 1993, 1997).

Although increased sensitivity and reproducibility have also been reported for pulsed potential cleaning at carbon electrodes by several researchers (Berger, 1985; Ewing, Dayton, and Wightman, 1981; Fleet and Little, 1974; Tengyl, 1984; Van Rooijian and Poppe, 1991), those electrodes have not generally been successful for the detection of polar aliphatic compounds. This effect is attributable to the absence of appropriate electrocatalytic properties of carbon surfaces to support the anodic oxygen-transfer reaction mechanisms of polar aliphatic compounds.

8.3 ELECTROCATALYSIS AT NOBLE METAL ELECTRODES

The majority of easily detected compounds at solid anodes under constant applied potentials are self-stabilized via π -resonance. Therefore, a desirable characteristic of electrodes in dc amperometry is inertness. The electrode serves as a sink to provide and remove electrons with no direct involvement in the reaction mechanism. Since π -resonance does not exist in polar aliphatic compounds (e.g., carbohydrates), stabilization of reaction intermediates is actively achieved via adsorption at “clean” noble metal electrodes. Faradaic processes that benefit from electrode surface interactions are described as “electrocatalytic.” Unfortunately, an undesirable consequence of this approach is the accumulation of adsorbed carbonaceous materials, which eventually foul the electrode surface.

Noble metals (i.e., Pt and Au) are commonly considered to be inert, but under electrochemical conditions these electrodes are quite active. Figure 8.1A shows the current-potential (*i*-*E*) plot for an Au rotating disk electrode (RDE) in 0.1 *M* NaOH with (.....) and without (—) dissolved O₂. The anodic signals for the positive scan correspond to the formation of surface oxide (wave a) and the breakdown of water to O₂ (wave b). The cathodic peak on the following reverse scan corresponds to the cathodic dissolution of the surface oxide (wave c). If dissolved O₂ is present, a cathodic wave (wave d) is observed during the positive and negative scans. Except for hydrogen adsorption, reduction, and oxidation at potentials less than -500 mV, all other features of Pt electrodes are similar to that of Au electrodes in 0.1 *M* NaOH (see Figure 8.1B). Note that wave d for O₂ reduction is well resolved from wave a for an Au electrode, whereas, there is significant overlap of O₂ reduction and oxide formation for a Pt electrode. This difference accounts for the general preference of Au over Pt electrodes for the majority of PED applications.

From the voltammetric data, it is apparent that surface oxide is reversibly formed and dissolved by the application of alternating positive and negative potentials, respectively. Oxide-free, or clean, noble metal surfaces have an affinity to adsorb

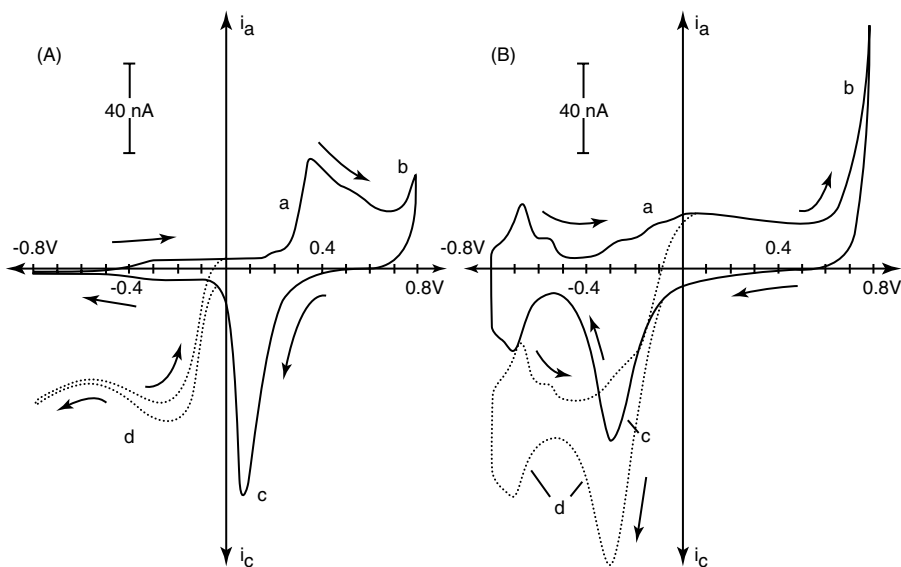


FIGURE 8.1 Cyclic voltammetric response (*i*-*E*) for (A) Au and (B) Pt RDE. Conditions: rotation speed, 900 rpm; scan rate, 200 mV s⁻¹; Ag/AgCl reference electrode. Solutions: (—) 0.1 M NaOH, deaerated; (.....) 0.1 M NaOH. (Reprinted from LaCourse, W.R., *Analysis*, 21, 181, 1993. With kind permission of Elsevier Science.)

organic compounds. Upon changing to a more positive potential, electrocatalytic oxidation of adsorbed compounds is promoted via anodic oxygen-transfer from H₂O by transient, intermediate products in the surface-oxide formation mechanism (i.e., AuOH and PtOH). Any fouling, which results as a consequence of the catalytic detection process or adsorbed compounds, is oxidatively desorbed from the electrode surface by application of a large positive potential, causing the formation of a stable surface oxide (i.e., AuO and PtO). These surface oxides are quite inert and must be cathodically dissolved by a negative potential excursion to restore the native reactivity of the oxide-free metal surface.

The three steps, (1) electrocatalytic oxidation for detection, (2) oxide formation to clean the electrode surface, and (3) oxide removal to reactivate the electrode and preadsorb analyte for the next cycle, form the basis of all PED techniques. Three modes of anodic electrocatalytic detection can occur at noble metal electrodes.

1. **Mode I: Direct Detection at Oxide-Free Surfaces.** At potentials less than ca. +200 mV (Figure 8.1A), oxidation of the compound can occur with little or no concurrent formation of surface oxide. The surface-stabilized oxidation results in a product which may leave the diffusion layer, reabsorb for further oxidation, or foul the electrode surface. The baseline signal originates primarily from double-layer charging, which decays quickly to a virtual zero value. All alcohol-containing compounds (e.g., carbohydrates) are detected by Mode I at Au electrodes in alkaline solutions and at Pt electrodes in acidic solutions (Austin et al., 1984; Edwards and Haak,

1983; Edwards et al., 1987; Hughes and Johnson, 1981–1983; Hughes, Meschi, and Johnson, 1981; Neuburger and Johnson, 1987; Olechno et al., 1987; Rocklin and Pohl, 1983).

2. **Mode II: Direct Oxide-Catalyzed Detection.** In contrast to Mode I detections, Mode II detections require the concurrent formation of surface oxide. Hence, Mode II detections occur at potentials $> \text{ca. } +150 \text{ mV}$ (Figure 8.1A). Oxidation of preadsorbed analyte is the primary contributor to the analytical signal; however, simultaneous catalytic oxidation of analyte in the diffusion layer is not excluded. The oxidation products may leave the diffusion layer or foul the electrode surface. Readsorption of analyte and detection products is attenuated by the surface oxide. The background signal, resulting from anodic formation of surface oxide, is large and has a deleterious effect on quantitation in HPLC-PAD. Aliphatic amines and amino acids (Johnson et al., 1986; Polta and Johnson, 1983; Welch et al., 1989) are detected by Mode II at Au and Pt electrodes in alkaline solutions. Numerous sulfur compounds (Johnson and Polta, 1986; LaCourse and Owens, 1995; Ngoviatchai and Johnson, 1988; Polta and Johnson, 1986; Polta, Johnson, and Luecke, 1986) are also detected by Mode II at Au and Pt electrodes in both alkaline and acidic solutions.
3. **Mode III: Indirect Detections at Oxide Surfaces.** Essential to Mode I and Mode II detections is the preadsorption of the analyte at oxide-free surfaces at negative potentials prior to electrocatalytic oxidation of the analyte itself. Species which adsorb strongly to the electrode surface and are electroinactive interfere with the oxide formation process. Preadsorbed species reduce the effective surface area of the electrode surface, and the analyte signal originates from a suppression of oxide formation. Since the baseline signal results from anodic currents from surface-oxide formation at a clean electrode surface, a negative peak results. Detection as a result of the suppression of surface oxide formation is known as Mode III detection. Sulfur-containing and inorganic compounds have been detected by Mode III (Johnson and LaCourse 1992; LaCourse, 1993, 1997).

Most frequently, PED is applied at Au electrodes under alkaline conditions ($\text{pH} > 12$) (see Figure 8.1A). All aldehydes, including “reducing sugars,” are anodically detected (Mode I) during the positive potential excursion at the oxide-free surface in the region of $\text{ca. } -0.6 \text{ to } +0.2 \text{ V}$ (Mode I). Large anodic signals are obtained for alcohols, polyalcohols, and nonreducing sugars in the region of $\text{ca. } -0.3 \text{ to } +0.2 \text{ V}$ (Mode I) with an attenuated signal for most compounds from $\text{ca. } +0.2 \text{ to } +0.6 \text{ V}$. Nitrogen- and sulfur-containing compounds, for which a nonbonded electron pair is present, are adsorbed at oxide-free Au surfaces for $E < \text{ca. } +0.1 \text{ V}$ and can be anodically detected by oxide-catalyzed reactions during the positive scan or $E > \text{ca. } +0.1 \text{ V}$ (Mode II). Detections at $E > \text{ca. } +0.8 \text{ V}$ are not recommended because of the deleterious effects of evolution of O_2 . Although there is little or no individual compound selectivity, functional group selectivity is clearly evident. Electroinactive surface-adsorbing species can be detected by suppression of the oxide formation process (Mode III) at potentials $> \text{ca. } +0.2 \text{ V}$.

Voltammetric resolution of complex mixtures is futile, since, electrocatalytic-based detection of various members within a class of compounds is controlled primarily by the dependence of the catalytic surface state on the electrode potential rather than by the redox potentials (E^0) of the reactants. Therefore, general selectivity is achieved via chromatographic separation prior to electrocatalytic detection. This conclusion does not preclude limited selectivity from control of detection parameters.

8.4 DETECTION STRATEGIES

The detection of carbohydrates, which represents the vast majority of food applications, occurs at electrode surfaces that are virtually free of oxides (Mode I). Mode I detections are best implemented using PAD waveforms. The electrochemical nature of carbohydrates is best elicited via studying the voltammetric response of glucose.

8.4.1 MODE I: ALDEHYDE- AND ALCOHOL-CONTAINING COMPOUNDS

The current-potential (i - E) response is shown in Figure 8.2 for an Au RDE in 0.1 M NaOH with (.....) and without (----) dissolved O_2 . With the presence of glucose (—), a reducing sugar, an anodic wave is observed on the positive scan beginning at ca. -0.6 V (wave E). This wave corresponds to oxidation of the aldehyde group to the carboxylate anion in this alkaline media. A much larger anodic signal is obtained for the combined oxidations of the alcohol and aldehyde groups in the region of ca. -0.2 to $+0.4$ V (wave F). The anodic signal is attenuated abruptly during the positive scan with the onset of oxide formation (wave A). The signal for ca. $+0.4$ to $+0.6$ V (wave G) in addition to that for oxide formation results from the anodic desorption of adsorbed glucose and/or intermediate products simultaneously with the formation of surface oxide on the Au electrode. The absence of signal on the negative scan in the region of ca. $+0.8$ to $+0.2$ V indicates the absence of activity by the oxide-covered electrode surface. Following cathodic dissolution of the oxide on the negative scan to produce wave C, the surface activity for glucose oxidation is immediately returned and an anodic peak (H) is observed for oxidation of alcohol and aldehyde groups on glucose. Anodic waves E, F, G, and H are all observed to increase in signal intensity with increases in glucose concentration. Although PAD for carbohydrates can be performed at either Au or Pt electrodes in alkaline media, the use of Au electrodes has the distinct advantage that detection can be achieved without simultaneous reduction of dissolved O_2 (wave D, Figure 8.2). The residual (----) response characteristics correspond to those discussed in Figure 8.1A. Similar voltammetric response curves are obtained for all other carbohydrates, except that nonreducing carbohydrates will not show a wave commencing at -0.6 V corresponding to the oxidation of the aldehyde group.

Although similar electrode processes are involved in the oxidations of all carbohydrates, significant differences exist in their reaction dynamics (Van Rooij and Poppe, 1991). These differences are reflected in the shape of current vs. bulk concentration (i - C^b) plots. Calibration plots for sorbitol and glucose show an extended linear range as compared to calibration plots for sucrose and maltose. Fructose's response is intermediate to those of glucose and sucrose. Two tentative explanations

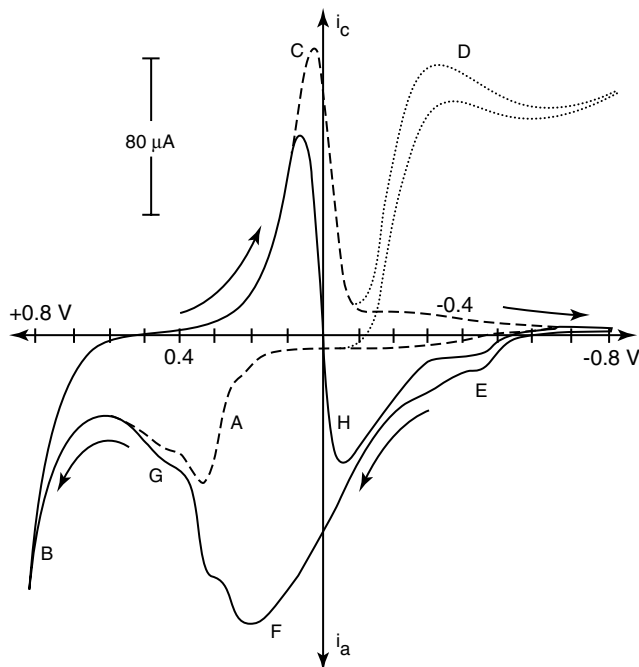


FIGURE 8.2 Cyclic voltammetric response (i - E) for glucose at an Au RDE. Conditions: rotation speed, 1000 rpm; potential scan, 200 mV s^{-1} ; Ag/AgCl reference electrode. Solutions: (.....) 0.1 M NaOH with dissolved O_2 ; (-----) 0.1 M NaOH, deaerated; and (—) 0.2 mM glucose, deaerated. (Reprinted from LaCourse, W.R. and Johnson, D.C., *Carbohydr. Res.*, 215, 159, 1991. With kind permission of Elsevier Science.)

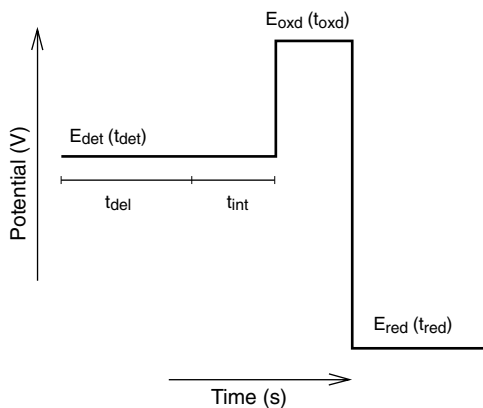
are offered for the nonlinear i - C^b plots for reactions characterized as being “under surface control.” First, the reactant molecules can be strongly adsorbed, and, therefore, response is controlled by the adsorption isotherm for that reactant. Second, if detection products are strongly adsorbed with the result of surface fouling, the current response will be attenuated more abruptly during the positive scan for large values of C^b for which full coverage of the surface is achieved more quickly. These effects necessitate the use of calibration curves to determine each carbohydrate’s range of linearity.

It is evident from Figure 8.2 that satisfactory results will be obtained for a rather wide range of detection potential (E_{det}) values. Since uniformly low levels of dissolved O_2 are difficult to maintain in HPLC systems, a choice of $E_{\text{det}} = +0.0$ to $+0.2 \text{ V}$ for the PAD waveform is a reasonable first estimate for universal detection of carbohydrates without interference from dissolved O_2 .

8.4.2 PULSED AMPEROMETRIC DETECTION

Oxide-free detections are implemented with a triple-pulse potential-time waveform (Table 8.1) at a frequency of ca. 2 to 0.5 Hz, which is appropriate for HPLC applications to maintain chromatographic peak integrity. E_{det} is chosen to be appropriate for the desired functional group, and the faradaic signal can be sampled during a short time (e.g., 16.7 ms) after a delay of t_{del} . Typical values of t_{del} are in the range

TABLE 8.1
PAD Waveform and Table of Waveform Parameters
for Carbohydrates in 0.1 M NaOH at an Au Electrode



Potential (mV vs. Ag/AgCl)			Time (ms)		
Parameter	General	Optimized	Parameter	General	Optimized
E_{det}	-200 to +400	+200	t_{det}	> 40	440
			t_{del}	> 20	240
			t_{int}	> 20	200
E_{oxd}	+300 to +800	+800	t_{oxd}	> 60	180
E_{red}	-800 to +100	-300/-800 ^a	t_{red}	> 60	360

^a $E_{\text{red}} = -300$ mV gives better RSDs, but it should only be used for relatively clean samples. $E_{\text{red}} = -800$ mV provides superior reductive cleaning.

of 100 to 600 ms. Following the detection process, the electrode surface is oxidatively cleaned by a positive potential step to E_{oxd} (+0.6 to +0.8 V) for t_{oxd} (50 to 200 ms) and then cathodically reactivated by a large negative step to E_{red} (-0.8 to -0.2 V) for t_{red} (100 to 600 ms) prior to the next detection cycle. Amperometric detection under the control of a three-step potential-time waveform is known as PAD.

Anodic detection of the aldehyde and alcohol functionalities in carbohydrates occurs in a potential region where there is only a very small background signal for the concurrent formation of surface oxide — Mode I. The development of positive peaks for carbohydrates and polyalcohols by Mode I in liquid chromatography (LC)-PAD is illustrated in Figure 8.3A by the chronoamperometric (i-t) response curves generated following the potential step from E_{red} to E_{det} in the PAD waveform. The residual current (curve a) decays quickly, and the baseline signal in HPLC-PAD is minimal for $t_{\text{del}} > \text{ca. } 100$ ms. Curve b in Figure 8.3A represents the transient i-t response for the presence of the reactant, and the peak shown is representative of the corresponding anodic signal expected in HPLC-PAD for the value of t_{del} indicated.

As with any detection system, an important consideration is the minimum detectable signal that can be recovered. The problem of differentiating an analytical

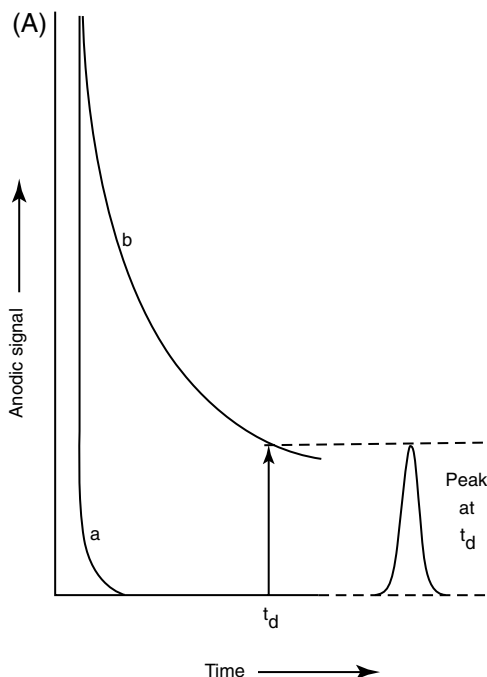


FIGURE 8.3 Chronoamperometric response (i-t) following a potential step from E_{red} to E_{det} in the PAD waveform without (a) and with (b) analyte present to illustrate the origins of chromatographic baseline and peak signals in LC-PAD for (A) Mode I and (B) Mode II detection. The delay prior to current sampling is denoted by t_d (i.e., t_{del}). (Reprinted from LaCourse, W.R. and Johnson, D.C., *Carbohydr. Res.*, 215, 159, 1991. With kind permission of Elsevier Science.)

signal from noise becomes increasingly difficult as the signal source becomes weaker (i.e., trace level concentrations). The ability of an instrument to discriminate between signal and noise is expressed in the signal-to-noise ratio (S/N) of the detection system. Any reduction in noise represents an increase in the S/N and an enhancement of the detection system.

The S/N for measurements of transient amperometric signals is influenced by the instrumental strategy used for sampling of the electrode current. A major noise component of the chronoamperometric signal is sinusoidal and correlated with a 60-Hz line frequency. Hence, a common strategy for current sampling in PAD involves some form of signal averaging over the period of one 60-Hz oscillation. Since there is no contribution to signal strength from the 60-Hz noise, the time integral of the 60-Hz sinusoidal noise signal (16.7 ms) is zero. Extension of this strategy to the integration of an integral number (m) of 16.7-ms periods results in a significant increase in analytical signal strength while maintaining a 60-Hz noise signal of zero. Typically, the integration period (t_{int}) is 200 ms, which is $m = 12$. Since 200 ms is an integral multiple of one period of 50-Hz oscillation (i.e., 20 ms), 50-Hz line noise is also minimized.

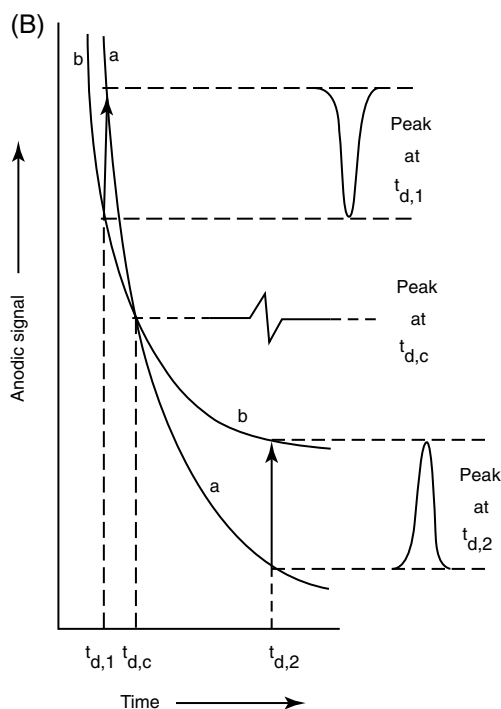


FIGURE 8.3 (continued)

8.4.2.1 HPLC-PAD and Waveform Optimization

The selection of potential values for the PAD waveform in HPLC-PAD has been discerned traditionally from the (i-E) response obtained for the analyte(s) of interest using cyclic voltammetry (CV). Cyclic voltammograms are generated using triangular potential-time (E-t) waveforms. LaCourse and Johnson (1993) have shown that the analyte response using CV, in which potential and time are coupled, is biased by the electrode surface at previous potential values in the scan. This effect is more noticeable for Mode I detections of strongly fouling compounds. In addition, the contributions of currents from oxide formation and double-layer charging are significantly different in CV vs. PAD. Aside from inappropriate potential selection of PAD waveforms from CV data, CV is virtually useless for determining optimum time parameters. PAD waveform optimization is best performed using a voltammetric response obtained when a PAD waveform is applied at a hydrodynamically controlled electrode (i.e., RDE) with small incremental changes in one of the parameters of the waveform (e.g., E_{det}) for each cycle of a multistep waveform. Such an approach is known as pulsed voltammetry (PV). PV has proven to be the definitive method for the optimization of PAD waveforms, especially for Mode I detections. Figure 8.4 shows the “background-corrected” $i-E_{\text{det}}$ response (positive scan direction) at an Au RDE for equimolar concentration of several carbohydrates in 0.1 M NaOH. As expected, the wave for the aldehyde group (glucose and maltose) begins at ca.

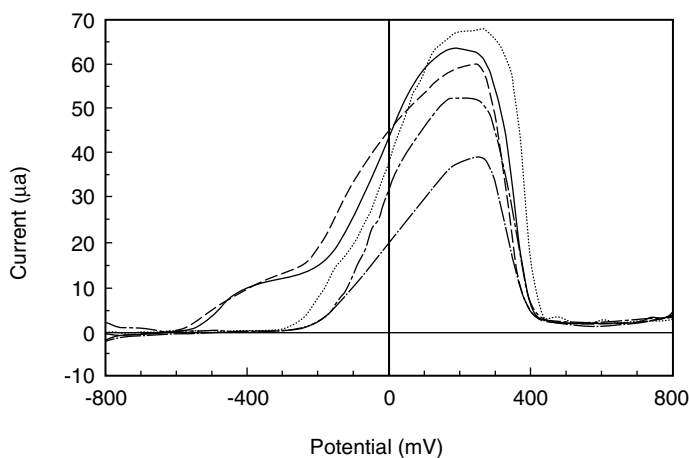


FIGURE 8.4 PV response for five carbohydrates at the Au RDE in 0.1 M NaOH. Solutions (0.1 mM): (.....) sorbitol, (—) glucose, (-----) fructose, (-·-·-) sucrose, and (----) maltose. (Reprinted with kind permission from LaCourse, W.R. and Johnson, D.C., *Anal. Chem.*, 65, 50–55, 1993. Copyright 1993 American Chemical Society.)

–600 mV and quickly plateaus. The peak signal in the region of –200 to +400 mV corresponds to the oxidation of the aldehyde group (glucose and maltose) and the alcohol groups (all carbohydrates). The response for all carbohydrates is inhibited by the formation of surface oxide (not shown) at ca. +400 mV; hence, carbohydrates represent oxide-free detections. A maximum response is obtained at $E_{\text{det}} = +180$ mV for all carbohydrates, and the application of this value is universal and results in the highly sensitive detection of virtually all carbohydrates. The advantage of PV is clearly evident when one compares the PV responses in Figure 8.4 with the CV plot of glucose in Figure 8.2. Table 8.1 lists the acceptable ranges and optimized value of E_{det} for the PAD waveform of glucose at Au in 0.1 M NaOH. In addition to peak amplitudes, S/N plots can be obtained using PV. A detailed description of PV has been published (LaCourse and Johnson, 1993).

8.4.2.2 Effect of pH and Organic Modifiers

Since carbohydrates are readily separated based upon anion formation in alkaline media on anion-exchange columns (Paskach, Lieker, and Thielecke, 1991), chromatographic separations are controllable via changes in pH, ionic strength, and organic modifiers. The consideration of pH-gradient elution must recognize the effect of pH change on the background signal as well as the choice of E_{det} for maximum sensitivity in HPLC-PAD (LaCourse, Mead, and Johnson, 1990; Mead, Larew, and Johnson, 1989). The potential for onset of oxide formation at Au electrodes shifts to more negative values with increases in pH at a rate of ca. -60 mV pH^{-1} . The effect of pH on the oxide formation process is attributable to the pH-dependent nature of Au oxide formation, i.e., $\text{Au}(\text{H}_2\text{O})_{\text{ads}} \rightarrow \text{Au-OH} + \text{H}^+ + \text{e}^-$. Because the optimal choice of E_{det} corresponds approximately to the value for onset of oxide

formation, values of E_{det} should be adjusted by the amount -60 mV pH^{-1} from the value of $+200 \text{ mV}$ recommended for 0.1 M NaOH . The negative shift in oxide formation with increasing pH can be reflected by a large baseline change in LC-PAD under pH-gradient elution when E_{det} remains constant throughout the gradient (LaCourse, Jackson, and Johnson, 1989). This effect can be alleviated to a great extent by substitution of a pH-sensitive glass-membrane electrode for the Ag/AgCl reference electrode in the PAD cell. Because the response of the glass-membrane electrode is ca. -60 mV pH^{-1} , the value of E_{det} is automatically adjusted during execution of pH gradients. Under ionic strength conditions suitable for ED (i.e., $\mu > 50 \text{ mM}$), the effect of changing ionic strength is reflected as minor perturbations in the background signal from oxide formation. This effect is not noticeable under isocratic HPLC conditions. Under gradient conditions (e.g., increasing acetate concentration), both positive and negative baseline drifts have been observed. In comparison to ionic strength effects, changes in the concentration of organic modifiers can have a much greater effect on the baseline signal in HPLC-PAD (LaCourse and Johnson, 1990). This can occur, even for electroinactive organic additives, because the modifiers are frequently adsorbed at the electrode surface with a resulting suppression of the oxide formation process (Mode III). In addition to alteration of the HPLC-PAD baseline, adsorbed organic modifiers can severely attenuate the analytical signal for carbohydrates by interfering with access to specific adsorption sites on the electrode needed for the reaction to occur.

8.4.3 MODE II: AMINE- AND SULFUR-CONTAINING COMPOUNDS

In addition to carbohydrates and alcohols, compounds which also have amine and/or sulfur moieties (e.g., aminoalcohols, aminosugars, thiosugars) rely on the detection of the $-\text{OH}$ groups to take advantage of the simplicities of PAD waveforms for Mode I detections. The amine and/or sulfur moieties are exploited to increase the adsorption of the analyte to the electrode surface. Strong adsorption of reacting molecules is considered very beneficial because the residence time of the molecule on the electrode surface is increased substantially, thereby increasing the probability for a successful faradaic reaction. In the case of compounds containing only amine and/or sulfur groups, only Mode II detections are often available.

8.4.3.1 Voltammetry

Although amine compounds can be detected directly at Pt (Johnson, 1986) and Au (Dobberpuhl and Johnson, 1995; Welch et al., 1989) electrodes only under alkaline conditions, numerous organic and inorganic sulfur-containing compounds can be detected across the entire pH range at both electrode materials. Both functional groups are detected by the oxide-catalyzed mechanism of Mode II. The Au electrode is preferred over the Pt electrode to minimize interference from dissolved O_2 .

The current-potential (i - E) response is shown in Figure 8.5A for an Au RDE in 0.1 M NaOH with (.....) and without (----) dissolved O_2 . With the presence of 0.2 mM lysine (—), an anodic wave is observed on the positive scan beginning at ca. $+0.1 \text{ V}$ (wave E). This wave corresponds to oxidation of the amine moiety. It is highly

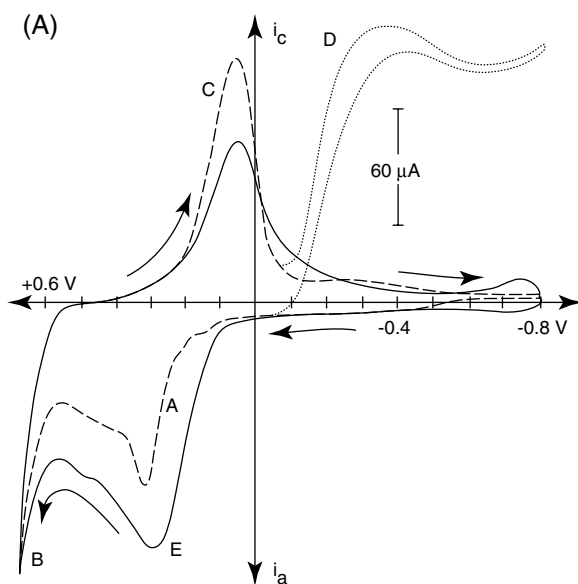
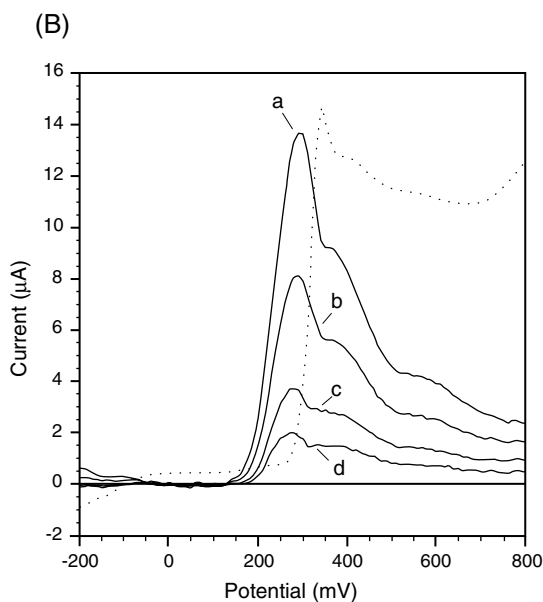


FIGURE 8.5 Current-potential (i - E) response curves for lysine at an Au RDE by (A) cyclic and (B) pulsed voltammetry. Conditions: rotation speed, 1000 rpm; potential scan, 200 mV s^{-1} ; Ag/AgCl reference electrode. Solutions: (.....) 0.1 M NaOH with dissolved O_2 ; (----) 0.1 M NaOH, deaerated; and (—) 0.2 mM lysine, deaerated. Pulsed voltammetric responses of lysine are background corrected. Conditions: rotation speed, 900 rpm. Waveform: E_{det} , variable; t_{det} , 450 ms; t_{del} , 240 ms; t_{int} , 200 ms; E_{oxd} , +800 mV; t_{oxd} , 180 ms; E_{red} , -800 mV, t_{red} , 360 ms. Solutions: (—) lysine, (a) $100 \mu\text{M}$, (b) $50 \mu\text{M}$, (c) $20 \mu\text{M}$, and (d) $10 \mu\text{M}$; (.....) 0.1 M NaOH, deaerated.

significant that lysine is detected only during the positive potential scan when surface oxide is being generated. No signal is observed for the negative scan when oxide growth has ceased. Hence, dc amperometric detection in the range of ca. +0.2 to +0.9 V is not expected to produce useful analytical signals at an Au electrode covered by inert oxide. Anodic wave E is observed to increase in signal intensity with increases in lysine concentration. All other amines and amino acids display similar voltammetric behavior with that of lysine. Reduction of dissolved O_2 occurs for $E < \text{ca. } -0.1 \text{ V}$ (wave D) during both the positive and negative scans. Figure 8.5B shows pulsed voltammograms of lysine at an Au electrode in 0.1 M NaOH, which is in general agreement with the CV of lysine (Figure 8.5A). Note that the majority of the analytical signal is in the region of ca. +180 to +500 mV, which corresponds to the oxidation of the amine group. Although the signal is present over the range of ca. +500 to +800 mV, it quickly decays, and the “inert” oxide layer on the electrode surface predominates the total current. Voltammetrically, amines behave similarly to amino acids. Therefore, the guidelines which correspond to amino acid detection apply equally well to that of aliphatic amines.

Numerous organic and inorganic sulfur compounds are adsorbed at the oxide-free surfaces of Au and Pt electrodes and can be detected by Mode II (Johnson

**FIGURE 8.5 (continued)**

and Polta, 1986; Ngoviatchai and Johnson, 1988; Polta, Johnson, and Leucke, 1986; Vandeberg, Kowagoe, and Johnson, 1992). These compounds include thioalcohols, thioethers, thiophenes, thiocarbamates, organic thiophosphates, and numerous inorganic compounds. Adsorption is a prerequisite to detection, and therefore, at least one nonbonded electron pair must reside on the S-atom. The kinetics for detection of adsorbed S-compounds are quite favorable at pHs from 0 to 14. Since alcohol and amine groups are detected only under highly acidic and/or alkaline conditions, the detection of sulfur compounds under mildly acidic conditions is highly selective. In addition, these pH conditions are compatible with reversed-phase separations.

The pulsed voltammetric response is shown in Figure 8.6 for an Au RDE in 250 mM acetate buffer (pH at 3.75) with (—) and without (.....) penicillamine. With the presence of (a) 50, (b) 20, and (c) 10 mM penicillamine, an anodic wave is observed on the positive scan at ca. +300 to +1500 mV. This wave corresponds to the oxidation of the thiol and, to a limited degree, the amine moiety. The lack of response of the amine moiety is deduced from the facts that the amine moieties are protonated at this pH, and valine shows little or no response under these same conditions. The anodic wave is observed to increase in signal intensity with increases in penicillamine concentration. The adsorption of sulfur-containing compounds, including penicillamine, is so strong that the onset of oxide formation is pushed to higher potentials. An artifact of the subtraction of the unperturbed residual response (.....) from the analyte response with an altered background results in the negative dip at ca. +1050 mV. This perturbation of the oxide in the presence of analyte leads to deleterious consequences (e.g., negative peaks) when using PAD waveforms. In

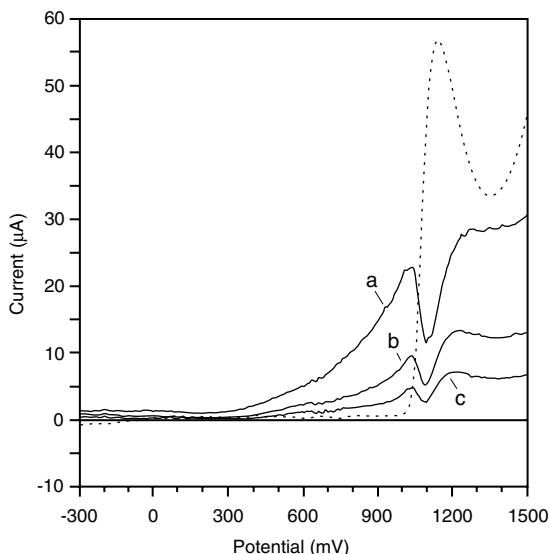


FIGURE 8.6 Pulsed voltammetric response of penicillamine at an Au RDE in 250 mM acetate buffer, pH 3.75. This plot is background corrected. Conditions: rotation speed, 900 rpm. Waveform: E_{det} , variable; t_{det} , 450 ms; t_{del} , 240 ms; t_{int} , 200 ms; E_{oxd} , +1500 mV; t_{oxd} , 180 ms; E_{red} , -300 mV; t_{red} , 360 ms. Solutions: (—) penicillamine, (a) 50 μM , (b) 20 μM , and (c) 10 μM ; (····) residual, deaerated. (From LaCourse, W.R. and Dasenbrock, C.O., *Advances in Chromatography*, Brown, P.R. and Gruska, E., Eds., Marcel Dekker, New York, 1997. Courtesy of Marcel Dekker.)

general, all sulfur compounds give very similar voltammetric profiles, except for the degree of perturbation of the residual response.

8.4.4 INTEGRATED PULSED AMPEROMETRIC DETECTION

Anodic detection of amine- and sulfur-containing compounds occurs in a potential region where there is a significant signal for the concurrent formation of surface oxide. Oxide-catalyzed detections are denoted as “Mode II.” The origins of detection peaks in LC-PAD based on Mode II are illustrated in Figure 8.3B. Here, the residual *i-t* response (curve a) corresponds to double-layer charging and to the formation of surface oxide. The current from surface-oxide formation decays much more slowly than the current from double-layer charging. Baseline signals for Mode II typically have a nonzero value over a large range of t_{del} values (see Figure 8.3B, curve a). The *i-t* response corresponding to the presence of adsorbed analyte is represented as curve b in Figure 8.3B. For small $t_{\text{d},1}$ (i.e., $t_{\text{del},1}$), “negative” peaks can be obtained in HPLC-PAD because of initial inhibition of the oxide formation process by the adsorbed analyte. For larger values of $t_{\text{d},2}$ (i.e., $t_{\text{del},2}$), “positive” peaks are obtained when sufficient oxide has been produced to catalyze the anodic reaction of the adsorbate. In the case of an unfortunate choice of an intermediate value of $t_{\text{d},c}$ at the crossover point, a detection peak might not be obtained. Choice of $t_{\text{del}} > \text{ca. } 150 \text{ ms}$ of a PAD waveform usually is sufficient to assure positive HPLC-PAD peaks based on Mode II.

TABLE 8.2
Disadvantages of PAD for Oxide-Catalyzed Detections

Baseline/background sensitivities

pH	Any changes or gradients in any of these variables will lead to baseline drifting.
Organic modifiers	The baseline drift is attributable to variations in the extent of surface-oxide formation
Ionic strength	
Temperature	

Sample-related effects

Poor S/N	The sample current is only a fraction of the total signal. The majority of signal for Mode II detections is derived from surface-oxide formation. The oxide formation signal tends to be noisy.
Postpeak “dips”	The presence of the analyte at the electrode surface interferes with surface-oxide formation. Hence, the background is different in the presence and absence of the analyte, which often results in a dip after the chromatographic peak.

As discussed for Figure 8.3B, a large baseline signal is encountered in HPLC-PAD for the oxide-catalyzed detections of amino acids and sulfur compounds (Mode II). Furthermore, the large baseline current is frequently observed to drift to large anodic values, especially for new or freshly polished electrodes. This drift is the consequence of a slow growth in the true electrode surface area as a result of surface reconstruction caused by the oxide on–off cycles in the applied multistep waveforms. As listed in Table 8.2, Mode II detections performed with PAD are subject to a number of disadvantages due to the formation of surface oxide, which is required and concomitant with the detection of amine- and sulfur-based compounds.

The disadvantages listed in Table 8.2 are either alleviated or greatly diminished by the use of the waveform in Table 8.3. Here, the electrode current is integrated electronically throughout a rapid cyclic scan of the detection potential (E_{det}) within the pulsed waveform. The potential scan proceeds into (positive scan) and back out of (negative scan) the region of the oxide-catalyzed reaction for detection by Mode II. The anodic charge for oxide formed on the positive sweep tends to be compensated by the corresponding cathodic charge (opposite polarity) for dissolution of the oxide on the negative sweep. Hence, the “background” signal on the electronic integrator at the end of the detection period can be virtually zero and is relatively unaffected by the gradual change of electrode area. IPAD combines CV with potential pulse cleaning to maintain uniform electrode activity.

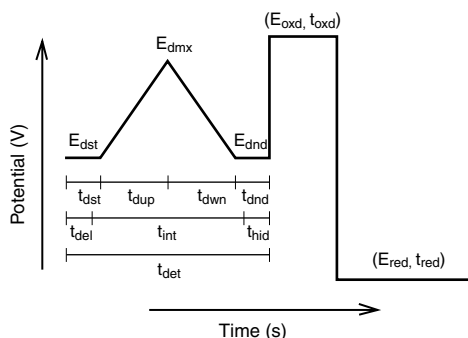
8.4.4.1 Waveform Optimization

There are several requirements pertaining to the cyclic sweep of E_{det} in IPAD which must be satisfied to achieve maximum success for applications to LC:

1. The cyclic scan of E_{det} must begin (E_{dst}) and end (E_{dnd}) at a value for which the electrode is free of surface oxide, i.e., $E < \text{ca. } +0.1 \text{ V vs. SCE}$ for Au in 0.1 M NaOH (see Figure 8.1A).

TABLE 8.3

Optimized IPAD Waveform for Thiocompounds and Summary of Parameter Functions



Parameter	Description	Acetate Buffer, pH 5.5E (mV),t (ms)
E_{dst}	Starting potential of scan, prior to oxide formation	-350
E_{dmx}	Maximum potential of scan for analyte oxidation	1500
E_{dnd}	Ending potential of scan, more negative than oxide dissolution	-350
E_{oxd}	Oxidation potential to initiate formation of oxide	1700
E_{red}	Reduction potential to initiate dissolution of inert oxide	-650
t_{dst}	Time at E_{dst}	20
t_{dup}	Time for scan up from E_{dst} to E_{dmx}	218
t_{dwn}	Time for scan down from E_{dmx} to E_{dnd}	218
t_{dnd}	Time at E_{dnd}	0
t_{det}	Total time of detection step	456
t_{del}	Delay time needed to overcome double layer charging	20
t_{int}	Integration time: signal collection/background rejection	436
t_{hid}	Hold time to complete oxide dissolution at E_{red}	0
t_{oxd}	Time at E_{oxd} to achieve complete formation of oxide	100
t_{red}	Time at E_{red} to achieve complete dissolution of inert surface oxide	200

- The value of E_{dnd} should not extend into the region for cathodic detection of dissolved O_2 , i.e., $E < -0.1$ V vs. SCE (see Figure 8.1A) if dissolved O_2 is present.
- The positive scan maximum (E_{dmx}) must not extend beyond the value for anodic solvent breakdown, i.e., ca. +0.7 V vs. SCE (see Figure 8.1A). When the majority of signal in an oxide-catalyzed (Mode II) detection can be found at the onset of oxide formation where analyte coverage vs. rate of oxide formation is most optimal, a greater S/N can be achieved by not scanning to the possible limit of this criterion.

It is to be noted from the residual i-E curve for Au in Figure 8.1A that only a small potential region centered at ca. -0.1 V vs. SCE is appropriate to satisfy criteria

(1) and (2) in 0.1 M NaOH containing dissolved O₂. The purging of solvents with He and the use of O₂-impermeable tubing can result in virtually O₂-free conditions which greatly relaxes constraints resulting from criteria 1 and 2.

At present, the IPAD waveform should be optimized utilizing the criteria presented previously in relation to CV data and using PV to optimize the pulsed potential steps as described for optimizing PAD waveforms. Table 8.3 lists optimized IPAD waveform parameters under one pH condition. Currently, work is underway to develop an IPAD optimization program similar to that of PV.

It is stressed that the advantage of IPAD, in comparison to PAD, relates to minimization of baseline drift for oxide-catalyzed detections (Mode II). Comparisons of IPAD with PAD for carbohydrates at the oxide-free surfaces (Mode I) have indicated virtually no significant difference between the two techniques, and the continued use of PAD for carbohydrate and alcohol detections is recommended.

8.4.4.2 HPLC-IPAD

A typical gradient system for the separation of a complex mixture often requires the combination of pH, organic modifier, and ionic strength gradients. The current produced by analyte detection based on Mode II is accompanied by current from surface-oxide formation. Consequently, variables (e.g., pH, organic modifier, and ionic strength) which affect the rates of oxide formation and dissolution will be reflected as drift in the baseline in HPLC-PAD. For these reasons, IPAD was designed to apply a waveform which coulometrically rejects the oxide background by summing the charges due to oxide formation and oxide dissolution which are expected to be of equivalent magnitude but opposite polarity. IPAD can virtually eliminate drift and changes associated with variations in composition (e.g., ionic strength and organic modifier) of the mobile phase, as well as changes in the total surface area of the noble metal electrode surface. Changes in pH may require the simultaneous use of a pH reference electrode (LaCourse and Johnson, 1990; LaCourse, Mead, and Johnson, 1990).

8.5 REVIEW OF FOOD APPLICATIONS

Since the inception of PED, the food industry has been both a pioneer and a benefactor of this technology. The success and acceptance of PED for the analysis of foods and beverages is indicated by the large number of PED-related publications and officially approved methods by regulatory agencies. Officially approved methods include:

- ISO 11292 — *Instant Coffee: Determination of Free and Total Carbohydrates — Method by High-Performance Anion-Exchange Chromatography* (International Standards Organization)
- ICUMSA — *Determination of Molasses* (International Commission for Uniform Methods of Sugar Analysis)

The following summary of food applications covers the history, breadth, and strong analytical utility of PED techniques for food and beverage analysis.

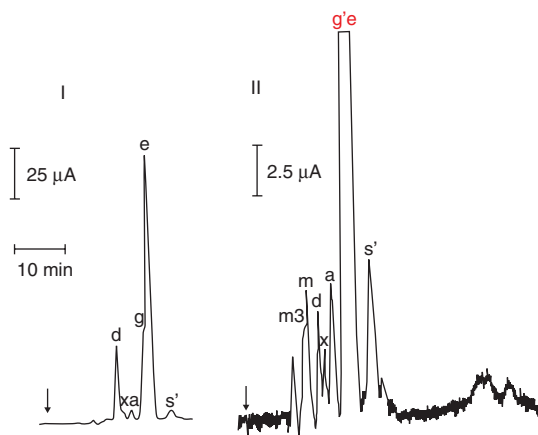


FIGURE 8.7 Chromatographic analysis of (I) Miller Lite beer and (II) Coors beer. Peak identities are m3 = maltotriose, m = maltose, d = dextrose, x = xylose, a = arabinose, g = glycerol, e = ethanol, and s = sorbitol. Chromatographic conditions: Ca(II) loaded cation-exchange column (Hamilton) maintained at 85°C, with a water mobile phase at a flow rate of 0.5 ml/min. Post column addition of base was necessary so 6.1 M NaOH was delivered at 0.01 ml/min. The detector was constructed in-house with a Pt wire working electrode, a Pt auxiliary electrode, and a saturated calomel reference electrode. (Reprinted from Hughes, S. and Johnson, D.C., *J. Agric. Food Chem.*, 30(4), 712–714, 1982. Copyright 1982 American Chemical Society.)

8.5.1 CARBOHYDRATE APPLICATIONS

8.5.1.1 Beverages

Hughes and Johnson (1982) describe the earliest carbohydrate analysis of beverages using PAD detection in which they quantified five components in different brands of beer. This work demonstrated the superiority of PAD combined with anion-exchange chromatography for carbohydrate analysis over refractive index detection using real samples. Samples were prepared by passing them through a solid-phase extraction (SPE) cartridge with a mixed-bed resin prior to analysis by cation-exchange chromatography-PAD with postcolumn addition of NaOH. In Figure 8.7, chromatograms showing the separation and detection of a light beer and a regular beer are shown. To demonstrate the versatility of this technique, carbohydrates in ginger ale and coconut milk were also determined. In each case, PAD provided a fast, direct analysis with few matrix peaks discernable.

Beer was also analyzed by Larew, Mead, and Johnson (1988) for the purpose of determining the total carbohydrate content in beer. In a flow-injection scheme, an enzyme reactor is added before the PAD detection step to facilitate the detection of oligosaccharides which do not have the high response factors that monosaccharides have. The glucoamylase reactor converts the disaccharides and oligosaccharides to glucose, and the total carbohydrate content is calculated from the glucose peak. This method also simplifies the quantitation because only one calibration curve is

required. Light beer and regular beer are compared to show the application of the enzyme reactor. The percent carbohydrate present in the light beer is known and compared favorably with the experimentally determined value.

Soga, Inoue, and Yamaguchi (1992) analyzed the carbohydrate and sugar alcohol content of beer using hydrophilic interaction chromatography with postcolumn addition of NaOH to facilitate PAD detection. For the separation, an acetonitrile (ACN)–water mobile phase was used. Determination of the monosaccharide and oligosaccharide profiles of beer during various stages in the brewing process was performed to show the applicability of HPAEC-PAD to the quality control of beer at various stages in the brewing process, from the starting materials to the final product (Dionex Technical Manual, 1995).

Wine was analyzed by Hughes and Johnson (1983) and carbohydrates and alcohols were determined. Cation-exchange chromatography with a water mobile phase was used for the separation, and postcolumn addition of NaOH was necessary for PAD detection. Both dry and sweet wine was analyzed, and, as expected, the dry wine had much less fructose and dextrose than the sweet wine. A peak for sorbitol was not observed for any of the wines tested. Because sorbitol is not naturally present in grapes, its presence in wine would indicate adulteration of the wine with other fruit juices.

Bernarl et al. (1996) analyzed wines made in different regions by HPAEC-PAD in order to detect differences in the grapes used and in wine-making techniques. Sample cleanup consisted of elution through a C₁₈ SPE cartridge prior to analysis to remove the pigments present in red wine. White wine was not subjected to this process. Seven monosaccharides were determined in both red and white wines. Wines which had been fermented for 6 and 12 months were used in this study, and the results show the disappearance of the sugars with fermentation time as expected. Differences with respect to the winemaker were also evident, presumably because of varied wine-making techniques employed in the process.

Wine coolers and brandy were analyzed for aliphatic alcohols using ion exclusion chromatography-PAD with minimal sample preparation (LaCourse et al., 1991). In each case, glycerol and ethanol were found with methanol (MeOH) present in trace amounts.

Bernarl et al. (1996) also determined seven monosaccharides present in instant coffee. A C₁₈ SPE step was again used prior to analysis to protect the chromatography system. Two of the coffees used in this study were adulterated. This was easily detectable because the levels of glucose and fructose were elevated compared to the unadulterated coffees. Free carbohydrates and total carbohydrate content of soluble coffee was determined in a multiple laboratory study (Prodoliet, Bugner, and Feinberg, 1995). Eleven different laboratories analyzed six different coffee samples, and the results were compared to determine the precision of the HPAEC-PAD method in the different laboratories. When the carbohydrate content in the coffee was greater than 0.3%, the precision of the HPAEC-PAD method was excellent. In another study, carbohydrate content in instant coffee was determined by HPAEC-PAD (Dionex Technical Manual, 1995).

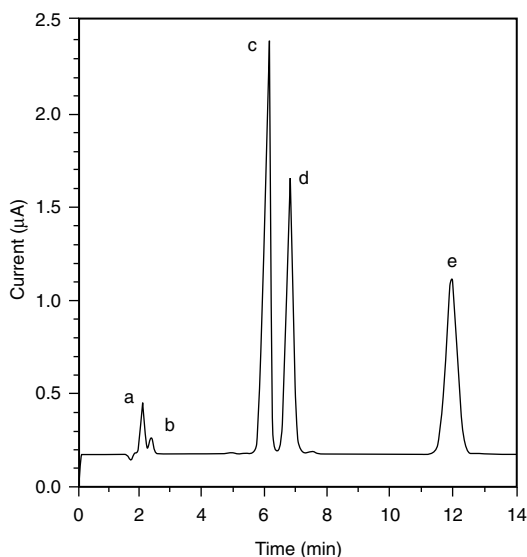


FIGURE 8.8 Chromatogram of carbohydrates in orange juice. Peak identities are a = xylitol, b = sorbitol, c = glucose, d = fructose, and e = sucrose. Chromatographic conditions: CarboPac PA1 anion-exchange column (Dionex), 100 mM NaOH mobile phase with a 1 ml/min flow rate; PAD at a gold electrode. (Reprinted with permission from Zook, C.M. and LaCourse, W.R., *Curr. Sep.*, 14(2), 48–52, 1995.)

8.5.1.2 Fruit Juices

Determination of carbohydrates in beverages has received some attention because it can be used for quality control of the juice, to identify cases of adulteration of the juice, and to aid in the determination of total carbohydrate content for labeling purposes (White and Widmer, 1990). Since PAD is selective for carbohydrates, the only sample preparation necessary is dilution and filtration. The primary source of carbohydrates present in the juices analyzed is glucose, fructose, and sucrose. Corradini and co-workers (1993) compared juices from apples, grapefruits, and oranges from different commercial sources and showed that the carbohydrate content was similar in each case. This work was followed up with a later paper in which fruit concentrates, juices, and nectars from apples, peaches, and pears were analyzed (Corradini, Cristalli, and Corradini, 1994). This work showed that the differences in the carbohydrate profiles between the juices are easily discerned by HPAEC-PAD and would be quite useful in the detection of fruit juice adulteration. White and co-workers (1990) compared the performance of PAD with refractive index detection (RID) for carbohydrate analysis. PAD compares favorably with RID because it is rapid, simple, and sensitive with detection limits 100 times more sensitive than that for RID. Juices from a number of different fruits were analyzed by Zook and LaCourse (1995) to demonstrate the application of PAD for determination of the carbohydrate content in foods and beverages. Figure 8.8 shows a separation of the

carbohydrates in orange juice. The carbohydrate content of fruit juices was shown to be an easy, routine analysis (Dionex Technical Manual, 1995).

Carbohydrate analysis of foods is an effective method of determining adulteration. Products such as juices can be manufactured more cheaply through the addition of inexpensive sweeteners such as high fructose corn syrup and invert sugar. Since PAD is selective and sensitive for carbohydrates, it is ideally suited for such a determination. Many adulterants used are quite similar to the fruit juice to which it is being added in order to avoid detection of the adulteration by regulators (Swallow, Low, and Petrus, 1991). Swallow, Low, and Petrus, 1991 and White and Cancalon (1992) present HPAEC-PAD methods for the determination of orange juice adulteration with beet medium invert sugar (BMIS). The oligosaccharide profile of the invert sugar is compared with that of the pure juice and the questionable juice. Even at levels as low as 5% BMIS added, the presence of adulteration is evident. White and Cancalon improved the method of Swallow and co-workers by reducing the run time from 71 to 31 min. These results are further supported by a similar study in which orange juice adulteration is determined by examination of the oligosaccharide profile (Dionex Technical Manual, 1995). Low and Wudrich (1993) also show that adulteration of fruit juice is readily determined by HPLC-PAD. All of these studies show that PAD is a simple, rapid, and direct method for the determination of carbohydrate profiles in juices and sweeteners.

Wang and Zopf (1989) determined oligosaccharides present in milk using ion-exchange chromatography followed by PAD. The eluted oligosaccharides were collected individually and subjected to acid hydrolysis. The monosaccharides were then determined using the same chromatographic system.

Lactose and sucrose were determined in a chocolate milk sample using HPAEC-PAD (Peschet and Giacalone, 1991). The sample was prepared by simply diluting 100 mg of chocolate milk in 1 l of water. The resulting chromatogram was very clean with the analyte peaks clearly detectable. No background peaks were observed. These studies demonstrate the ease of application of PAD to a very complex milk matrix.

8.6 FUNCTIONAL FOODS

Functional foods are a new concept in the United States. They are a rapidly emerging concept in the area of nutrition. They have become one of the most interesting areas of study, capturing the interest of not only the consumers but also of scientists (Clydesdale, 1997; Milner, 1999). The reason for this emerging interest is the widespread belief that certain foods and selected food components are associated with enhanced physical and mental performance along with reduced risk of disease (Craig, 1997; Messina and Messina, 1996). In this regard, the importance of functional foods can be attributed to Hippocrates and his now famous tenet, "Let food be thy medicine and medicine be thy food," a concept that goes back to ca. 400 B.C. (Hasler, 1996; Milner and Craig, 2000). Now almost 2500 years later, as we mark the beginning of a new millennium, the concept of food as medicine has taken on philosophical importance evolving into the concept of nutritional essentiality; certain foods and specific food components are essential for the health and survival of higher animal, thus forming the basis of functional foods. More recently, the relationships

between diet and disease have become major areas of investigation in nutrition, especially involving these functional foods (Harper, 1999).

Although functional foods have been around for some time, they seemed to have little impact on the consuming public at large. The consumer acceptance of functional foods is growing rapidly, and the markets for them have assumed global proportions. Functional foods are one of the largest and fastest growing consumer markets in Asia, Europe, and the United States. Japan, the birthplace of functional foods, has had for some time now the most developed and sophisticated consumer market for functional foods (Jago, 2000). To our knowledge, Japan is the only country with a formal regulatory approval process for functional foods. Officially designated as Foods for Specified Health Use (FOSHU), these functional foods have now come to bear the official seal of approval from the Japanese Ministry of Health and Welfare (Arai, 1996). With hundreds of products currently licensed as FOSHU foods, Japan is a good example of a successful and thriving functional foods industry and continues to provide leading-edge nutritional products. Generally speaking, all foods are functional foods in that they provide flavor, taste, aroma, and nutritional value. Nevertheless, more recently, the term functional food has come to mean one of providing a specific physiological benefit in addition to providing the basic nutritional requirements.

8.6.1 WHAT IS A FUNCTIONAL FOOD?

Despite their ubiquitous nature, functional foods have never been precisely defined. The term functional food was first introduced in Japan in the mid-1980s. Functional foods refer to processed foods containing significant levels of biologically active components that specifically impart health benefits or desirable physiological functions in addition to being nutritious. Other organizations have proposed definitions for functional foods. The Institute of Medicine's Food and Nutrition Board (IOM/FNB) defined functional foods as "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains" (IOM/NAS, 1994). Functional foods have received favorable reviews by the Functional Foods for Health (FFH) Program, a joint program of the University of Illinois at Chicago and the University of Illinois at Urbana-Champaign. In the United States, there is no legal recognition of the functional foods category. Health conscious consumers, especially the baby boomer generation, have provided the boost, making functional foods the leading trend in the U.S. food industry (Meyer, 1998). Other terms such as *dietary supplements*, *nutraceuticals*, *designer foods*, *phytochemicals*, and *food additives* have been presented to the consuming public. Here are some definitions. Dietary supplements, according to the Dietary Supplement Health and Education Act (DSHEA) of 1994, are defined as "products intended to supplement the diet to enhance health." These include vitamins, minerals, amino acids, herbs, and other botanicals. A dietary supplement is "not represented as a conventional food or a sole item of a meal or the diet." A nutraceutical was recently defined as a "diet supplement that delivers a concentrated form of a biologically active component of food in a nonfood matrix in order to enhance health" (Zeisel, 1999). Dietary supplements and nutraceuticals are distinct from functional foods, which deliver an active ingredient

within the food matrix, and from food additives, which enhance flavor or aroma without the nutritional value of a food.

One class of foods that falls under the broad category of functional foods is designer foods. A designer food is created by modifying the composition of a basic food product to incorporate certain nutrients or other bioactive compounds for a specific health purpose. A designer food may be modified by genetic engineering, hybridization techniques, or feeding specific diets to livestock or poultry. A phytochemical is a physiologically active, naturally occurring compound produced by plants that is not classified as a nutrient, but nevertheless provides a health benefit when consumed. A variety of phytochemicals have been discovered and are now available to the consuming public. Some designer foods have been modified to incorporate phytochemicals, and some phytochemicals are available in supplement form.

Another class of functional foods is the prebiotics and probiotics. A prebiotic is defined as a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon. An example of a prebiotic is the inulin-type fructans. Despite the limited work done with prebiotics, studies with these inulin-type fructans have generated sufficient data for their consideration as putative functional food components. A probiotic on the other hand is defined as a viable microbial dietary supplement that beneficially affects the host through its effects in the intestinal tract. Present examples of probiotics include fermented dairy products such as yogurt or freeze-dried cultures. Future examples of probiotics will include fermented vegetables and meats (Roberfroid, 2000). This brings one to the definition of nutrition.

According to *The Webster's New World Dictionary*, nutrition is defined as, "a series of processes by which an organism takes in and assimilates food for promoting growth and replacing worn or injured tissues." It follows that a nutrient is a substance in food that is essential for maintaining health. These include carbohydrates, proteins, lipids, vitamins, and minerals. Therefore, functional foods contain nutrients in the proper balance to not only help maintain health, but to also contain other compounds that promote and protect health.

There have been major conferences on functional foods as part of a new global awareness on the subject. The First International Conference on East-West Perspectives on Functional Foods was held in September 1995 in Singapore (*Nutr. Rev.*, 1996). The issues addressed were historical and cultural perspectives on functional foods, their role in disease prevention and health promotion, scientific and technological advances within different regions and countries, scientific data on the effects of various bioactive and nutritive food components on human physiological systems, selected case studies of specific foods or classes of foods, the safety of functional foods and substantiation of their putative health benefits, a discussion of an appropriate definition of functional foods, and regulatory issues. Recently, a conference was held in Copenhagen, Denmark in October 1998. This conference provided a global analysis of the scientific, commercial, and regulatory activities in North America, Western Europe, and the Pacific region in the area of functional foods and nutraceuticals. At the American Association of Cereal Chemists (AACC)-sponsored conference on Functional Foods: Strategies for the Food Industry, held in June 1999

in Newport Beach, CA, the meeting focused on the development and production of functional foods tailored specifically to the food industry. At the Annual Experimental Biology 2000 meetings, the Third Annual Symposium on Functional Foods for Health Promotion was held in April 2000 in San Diego, CA. This symposium, sponsored by International Life Sciences Institute of North America, focused on factors that affect the physiological responses to biologically active components in foods (*Nutr. Today*, 2000b). More recently, an International Conference and Exhibition on Nutraceuticals and Functional Foods was held in September 2000 in Houston, TX. This conference was sponsored by The International Union of Food Science and Technology.

The Institute of Food Technologist (IFT) recently published a *Scientific Status Summary* on functional foods (*Nutr. Today*, 2000a). This Scientific Status Summary reviewed the research to date for the primary plant and animal foods associated with physiological benefits. Although much has been written about bioactive compounds in this regard (Kuhn, 1998), the publication of the IFT focused on whole foods rather than specific components isolated from foods. The functional foods from plant sources included oat products, soy and soy products, flaxseed, tomatoes, garlic, broccoli and other cruciferous vegetables, citrus fruits, cranberries, green tea, wines, and grapes. The functional foods from animal sources included fish, dairy products, and beef.

The functional foods mentioned above received notoriety of sorts because of the rapid growth of consumer interest in the importance of their bioactive compounds. Lycopene, for example, the primary phytochemical found in tomatoes, has significant antioxidant potential *in vitro* and has been linked with preventing prostate cancer and cardiovascular disease in humans. Isoflavones in soy and lignans in flaxseed oil, acting as weak estrogens, may play a role in reduced risk of breast cancer. Garlic, containing organosulfur compounds, may have a role in lower blood pressure, reduced cancer risk, and other functions. Limonoids in citrus fruits, glucosinolates in cruciferous vegetables, and catechins in green tea appear to have protective roles in a variety of human cancers. Probiotics in fermented dairy products such as yogurt are associated with a reduced risk of colon cancer, low serum cholesterol, and other effects resulting from altered gut flora.

The rapidly growing consumer and public interest in functional foods has produced a great demand for information. In the United States, this interest in functional foods is fueled by the following factors: rising healthcare costs, rising interest in wellness programs through diet, an aging population, changes in federal food laws affecting product and label claims, and recent advances in science and technology. Attitudinal research done by the International Food Information Council (IFIC) Foundation shows that consumers today are much more interested in the concepts and principles of functional foods than many health professionals realize. To date, the Federal Food, Drug and Cosmetic Act (FFDCA) has not provided a formal federal statutory definition of functional foods. Therefore, the U.S. Food and Drug Administration (FDA) has no federal authority to formally regulate functional foods under the category of foods, including the expanded category of dietary supplements. Since the FDA regulates foods based on their intended use, this is determined largely by the label and label claims accompanying the product. Scientific research in food

sciences suggests many potential health benefits from food components. These health benefits could expand the health claims now allowed to be considered and identified by the FDA.

8.6.2 SCIENTIFIC CRITERIA FOR FUNCTIONAL FOODS

There is increasing scientific evidence suggesting that physiologically active components in functional foods, of either plant or animal origin, may have health benefits. This is supported by research efforts at a number of academic, scientific, and regulatory organizations with efforts underway to establish scientific criteria to help support the health claims for functional food components or the foods themselves. From a regulatory standpoint, two types of claims are allowed on foods and dietary supplements in compliance with FDA regulations: (1) structure/function claims describing effects on the normal function of the body and (2) health (or reduced disease risk) claims implying a relationship between diet or diet components and a health (or disease) condition as approved by the FDA and supported by significant scientific agreement. To narrow the gap between foods and dietary supplements, the FDA recently announced the creation of a new Office of Nutritional Products, Labeling, and Dietary Supplements. This replaces the Office of Special Nutritionals and the Office of Food Labeling. Nevertheless, it should be emphasized that functional foods are not a universal substitute for poor eating habits. Emphasis, however, must be on a balanced overall dietary model based on the U.S. Dietary Recommendations: one that is plant-based, low in animal fat, high in fiber, and consuming five to nine servings of fruits and vegetables.

Consumers are becoming increasingly health conscious, seeking out and incorporating functional foods into their diets in pursuit of good health maintenance. The area of functional foods is in its infancy and gaining popularity. Any health claims associated with functional foods must be based on sound scientific criteria (Clydesdale, 1997). In pursuit of this sound scientific principle, a large body of solid scientific research is needed to validate the potential benefits of any particular functional food or its component. In this regard, one must consider several factors, such as the complexity of the functional food matrix, effects on the functional food, compensatory metabolic changes accompanying dietary changes, and the need for biomarkers of disease development.

And last but not the least, the consuming public must have a clear understanding of, and a strong confidence in, the sound scientific criteria used to substantiate the health effects and claims regarding functional foods. The scientific community is only now beginning to understand functional foods and their ability to deliver potential health benefits. Until this knowledge is complete, consumers must be aware that functional foods are not magic bullets or a total panacea. Examples of functional food components include carbohydrates, carotenoids, fatty acids, flavonoids, glucosinolates, phenols, plant sterols, prebiotics, probiotics, saponins, soy proteins, sulfides, thiols, and tannins.

The following section will focus on carbohydrates in functional foods and HPAEC-PED applications, which were introduced earlier, to their analysis.

8.6.3 CARBOHYDRATES IN NUTRITION

As mentioned above, functional components are those ingredients that impart functionality to the functional food. This section will focus on reviewing the information about carbohydrates as specific food components. Before discussing the carbohydrates in functional foods, let us take a look at carbohydrates in food. The goal here is to explore the scientific literature on food carbohydrates in general with nutritional properties.

Carbohydrates as a food group contribute significantly to nutrition. Scientists have been studying for some time now how carbohydrates work individually and in concert with other food components to regulate physical and mental stamina and modulate stress. Since the physical and chemical structure of foods vary, calories are available from some foods sooner than from others. Food is fuel, and depending on the activity planned for the day, the food you eat should suit the occasion. A diet planned to include certain foods will help obtain and sustain the kind of specific energy a person needs for peak performance.

As energy-yielding compounds, carbohydrates together with proteins, fats, and a few minerals, represent the *sine qua non* of a nutritionally essential diet. In the United States, most people are waking up to the fact that a diet rich in plant foods is healthy and protective. The new Food Pyramid Guide introduced by the U.S. Department of Agriculture (USDA) (replacing the Basic Four Food Groups) reflects the modern nutritional wisdom in the United States. The role of carbohydrates in foods and their importance in nutrition has been discussed and debated in a recent article on healthy eating (Herman, 1997). The proponents maintain that a diet rich in high fiber and carbohydrates (along with low fat) is nutritionally healthy with high-fiber, complex carbohydrates comprising 60 to 75% of the diet (Herman, 1997). Opponents contend that fear of fat has led to an excessive, overconsumption of simple carbohydrates (refined sugar and white flour foods) rather than complex carbohydrates (whole grains, vegetables, and beans) (Herman, 1997). The debate goes on to mention that Americans have not reduced their fat intake and are loading up on the wrong kinds of carbohydrates. This leads to the next most important question. Are there good and bad carbohydrates?

A recent commentary in the *Lancet* suggests that there are (Katan, 1999). In one case study, the diets of over 65,000 women were examined over a 6-year period (Herman, 1997). Results from these studies showed that women who consumed a low-fiber diet along with plenty of soft drinks were two and half times more likely to get diabetes than those who consumed a high-fiber diet with very few soft drinks. This study showed that sugar was not the only culprit. Other dietary culprits included white bread, white rice, and potatoes. This finding led to the controversial Glycemic Index (GI) concept, which was developed as a tool to help people monitor and stabilize their blood sugar level.

Most processed foods contain considerable amounts of refined sugar and/or sweeteners. How has this impacted the nutritional quality of foods? A recent report entitled Changes in Nutritional Quality of Food Product Offerings and Purchases by the USDA's Economic Research Service indicates that the nutritional quality of foods has not improved (Chapman, 2000). This study analyzed the nutritional index

of five food categories. The specific nutrient content of certain foods showed significant changes. Processed meats and bacon had significant changes in carbohydrates, while in cookies the level of fiber improved. Increases in desirable nutrients were generally offset by increases in undesirable nutrients and vice versa.

Carbohydrates can be both a boon and a bane from a nutritional standpoint. For example, carbohydrates as mood-enhancing foods have long been recognized. Specifically, the carbohydrate connection to levels of serotonin, a brain chemical messenger that causes upbeat moods (positive changes in attitude and behavior), is well known (Turner, 1999). Research studies on the effects of serotonin levels in brain show that both simple sugars (candy, cookies, and honey) and complex carbohydrates (whole grains and most vegetables) cause upbeat moods in different ways. This is how it works: when carbohydrates are consumed, they trigger the release of insulin, which in turn increases the level of tryptophan, an amino acid in the brain. Tryptophan is then converted to serotonin, which is responsible for the upbeat mood of feeling well. There is one difference, however. Simple sugars produce positive mood shifts, mental function, and overall energy by providing empty calories in the short run, which is an apparent bane of the carbohydrate food. Complex carbohydrates on the other hand produce the healthy serotonin during high-stress times that provide the required nutrients instead of the empty calories, which is an apparent boon of the carbohydrate food.

The science of carbohydrates never tasted sweeter. It helps a consumer to understand that sugar is sugar is sugar. This is true for refined white sugar, maple syrup, or barley malt. Even though refined white sugar has come to be regarded as the culprit, sugar consumption in any form may not be good for anyone. For example, sugars help promote tooth decay and prevent the availability of the more nutritious foods. Excessive sugar consumption can be especially bad for those at risk for diabetes, obesity, hypoglycemia, or coronary heart disease. Consumer misconception about the so-called natural sweeteners is well known. Just because it says "natural," it does not mean that it is better for you. Some sweeteners are known to contain complex carbohydrates fortified with vitamins and minerals. With the exception of molasses, nutritionally speaking there is no advantage of one sweetener over the other (Cheney, 1995).

It is important to understand how the body processes sugar. Sugars are simple carbohydrates. They occur as monosaccharides (glucose, fructose, or galactose) or disaccharides (sucrose, lactose, or maltose). Simple carbohydrates are found naturally in milk, fruits, and vegetables, as well as in processed foods such as table sugar, candy, and soft drinks. Polysaccharides are complex carbohydrates composed of long glucose chains plus fiber and other nutrients. They are found in grains (cereal, rice, bread, and pasta), legumes (lentils, peas, and beans), and starchy vegetables (corn, potatoes, and peas). Regardless of whether it is a simple or complex carbohydrate, the primary dietary function of a carbohydrate is to provide energy. Enzymes of the digestive system hydrolyze all carbohydrates to the simple monosaccharide glucose, commonly referred to as the blood sugar, for energy (Cheney, 1995).

From the foregoing discussion, there is clear evidence that consuming large amounts of simple sugars on a regular basis puts a strain on our physiological systems. Because they are packed with fiber and nutrients, complex carbohydrates

are considered by many nutrition and health experts to be the foundation of a healthy diet (Quagliani, 1997). They are also considered the building blocks of food due to their broad functionality (Ohr, 1998). For example, research on starches and hydrocolloids have improved the quality of cereals, candy, and low-fat foods. Furthermore, starches and hydrocolloids are two of the widely known fat mimetics used in food. The Whistler Center for Carbohydrate Research at Purdue University in West LaFayette, IN is well known for their research studies on complex carbohydrates.

Nevertheless, the American Dietetic Association recommends that 55 to 60% of the total daily calories come from both simple and complex carbohydrates (Quagliani, 1997). This recommendation calls for a diet rich in simple carbohydrates found in nutrition-packed milk, vegetables, and fruits and allowing for higher fat and higher calorie sweets as well. The recommendation goes on to mention that the source for most of the carbohydrates should be the complex type.

The structure of carbohydrates has a major impact on their function in different applications. The variability of carbohydrate structures can be seen in their chain length, the type of carbohydrate units in the chain, and the position of glycosidic linkages that affect physicochemical properties. A Joint FAO/WHO Expert Consultation on Carbohydrates in Nutrition was held in Rome, Italy, April 1997 (FAO, 1998). This consultation was wide ranging in its scope and published the report essentially as a reference document. This report reviewed and outlined the state of the knowledge on the important roles of carbohydrates in human nutrition and health. The consultation discussions focused on the following: (1) the great strides made in carbohydrate chemistry in the development of a variety of new food products, many of which were based on nutritional considerations; (2) a growing understanding of the diverse physiological roles of carbohydrates in digestion and fermentation in the gut; (3) the influence of carbohydrates on physical performance through glycogen loading; (4) issues with important implications for agricultural production, the food industry, and public health policy; (5) formulation and implementation of science-based dietary guidelines for the nutritional sciences; and (6) the role of carbohydrates in noncommunicable diseases. This consultation reached several conclusions on each of the topics discussed and made a number of scientifically sound, up-to-date, pragmatic recommendations on carbohydrates in nutrition.

8.6.4 CARBOHYDRATES IN FOOD

An understanding of the carbohydrate functionality requires an understanding of the structural, chemical, and physical properties of carbohydrates. Carbohydrates in food comprise anything from a simple carbohydrate (simple sugar or monosaccharide) to highly complex carbohydrates (polysaccharides). Because each of these carbohydrates has certain properties, the structural diversity found in food carbohydrates offer different functional properties. The functional properties are generally derived from the carbohydrate content and are directly related to the carbohydrate structure. Let us take a look at the various carbohydrates found in food.

All carbohydrates are generated via the process known as photosynthesis from the sun's energy combined with carbon dioxide and water in the plant. Carbohydrates

are important organic compounds composed of carbon, hydrogen, and oxygen. Chemically, they are polyhydroxy aldehydes, ketones, alcohols, or acids. They have important functional roles both in plants and in the human body. Food carbohydrates come in two principal groups: *simple carbohydrates* and *complex carbohydrates*. Simple carbohydrates consist of any soluble, digestible, and absorbable sugars such as monosaccharides, disaccharides, and sugar alcohols or polyols. Complex carbohydrates comprise the oligosaccharides and polysaccharides (starches and dietary fiber).

Monosaccharides are the simplest sugars containing a single sugar unit. These include glucose, fructose, and galactose. Monosaccharides are the building blocks from which all the other carbohydrates are made. Two monosaccharide units link together to form disaccharides. The most common disaccharides are sucrose (glucose + fructose), lactose (glucose + galactose), and maltose (glucose + glucose). These monosaccharides and disaccharides are available as pure crystalline compounds for use as food ingredients. As a result, their functional properties follow a relatively predictable pattern. Sugar alcohols or polyols are produced by hydrogenating monosaccharides such as glucose, thereby transforming the reducing end of the glucose molecule to a hydroxyl (–OH) group. The most common sugar alcohols are sorbitol, mannitol, and xylitol.

Oligosaccharides contain anywhere from three to nine monosaccharide units. Oligosaccharides formed mainly from the hydrolysis of starch are known as maltooligosaccharides. Other oligosaccharides include raffinose (a trisaccharide), stachyose (a tetrasaccharide), and fructooligosaccharides (inulins).

Polysaccharides are made from linking multiple monosaccharide units. These long chain molecules are known as starch in the plant system and glycogen in the animal system. Starch polysaccharides primarily contain α -glucans. These include amylose, amylopectin, modified starches, and resistant starches. Fiber, also known as nonstarch polysaccharides, primarily contains components derived from the plant cell wall such as cellulose, hemicellulose, and pectin. Other polysaccharides include gums and hydrocolloids.

Now that the important food carbohydrates have been defined and identified, the next step is to classify these food carbohydrates as important dietary carbohydrates combining chemistry and nutrition. Efforts trying to reconcile the chemistry and nutritional aspects that best reflect the physiological properties have led to a terminology of dietary carbohydrates describing these carbohydrates and their various fractions and subfractions (FAO, 1988).

8.6.5 DIETARY CARBOHYDRATE TERMINOLOGY

8.6.5.1 Sugars

Sugars generally refer to the simple carbohydrates or sugars. These include the monosaccharides and disaccharides. On the other hand, sugar generally refers to table sugar (purified sucrose) and is also known as refined sugar. The terms *sugars* and *sugar* are used to inform the consumer between those that are considered healthy sugars and those that are not.

8.6.5.2 Complex Carbohydrates

First described in a report entitled “Dietary Goals for the United States” published in 1977 (FAO, 1998; U.S. Senate Select Committee on Nutrition and Human Needs, 1977), complex carbohydrates were used to mean *whole grains, fruits, and vegetables* and to distinguish them from sugars. More recently, complex carbohydrates have come to mean starch, dietary fiber, or non-digestible oligosaccharides. It is now realized that starch, a complex carbohydrate by any definition, occurs in various forms. These variable forms of starch are metabolized differently, with some forms of starch being readily absorbed and having a high GI and other forms being resistant to digestion. Complex carbohydrates are plentiful in grains (cereal, rice, pasta, and bread), starchy vegetables (corn, peas, and potatoes), and legumes (lentils, peas, and dried beans).

Dietary carbohydrates were further classified into *available* and *unavailable* carbohydrates (FAO, 1998). Available carbohydrate was defined as soluble sugars and starches, whereas unavailable carbohydrate was defined as mainly hemicellulose and cellulose (fiber). The concept of dietary carbohydrates described as available and unavailable was based on the two important properties of carbohydrates: digestibility and fermentability.

8.6.5.3 Resistant Starch

Resistant starch (RS) was defined as “starch and starch degradation products not absorbed in the small intestine of healthy humans” by Englyst and Cummings (1990). The finding of RS has added to our understanding of the important role carbohydrates play in human health. The different nutritional characteristics of starch, whether digested and absorbed readily or slowly or undigested, led to their nutritional classification as “readily digestible,” “slowly digestible,” or RS (Englyst, Kingman, and Cummings, 1992). The principal forms of RS are RS1 (physically trapped starch), RS2 (resistant starch granules), and RS3 (retrograded starch) (Cho, Prosky, and Dreher, 1999; Englyst and Cummings, 1990; Englyst, Kingman, and Cummings, 1992). RS is formed when starch or starch-containing foods are heated, for example, during bread baking. RS also has many characteristics of the fermentable dietary fiber and is important to both the consumer and the food processor in that it may provide the nutritional boost to the dietary fiber content of the food in question.

8.6.5.4 Modified Starch

Starch is the main carbohydrate component of many food products. Furthermore, starch and starch degradation products provide a wide range of functional properties. Starch is primarily composed of amylose and amylopectin. The proportions of amylose and amylopectin in a starch-containing food can vary. These variations can be brought about by food processing and genetic engineering techniques. For example, corn starch containing high amylose and high amylopectin has been available for some time now with different functional and nutritional properties. High-amylose starches gelatinize only at higher temperatures, are more susceptible to retrogradation, and have a tendency to form amylose–lipid complexes. These properties can be fully utilized in formulating foods with high resistant starch content.

Today, stringent demands are being placed on this important complex carbohydrate, starch, by the food industry. Not long ago, starch was seen as an unattractive collection of complex macromolecules lacking the fine challenging structural features. It is not only expected to behave as a super molecule with great physical and chemical attributes, but also to display unique functional as well as nutritional properties. Unfortunately, native starches do not measure up to such requirements and must be modified to improve their functional properties. Starches can be modified physically, chemically, or genetically (Eliasson and Gudmundsson, 1996). Physical modifications involve changes in starch structure or phase behavior and interaction between starch and protein or lipid components and do not involve covalent linkages. Specifically, these modifications include partial hydrolysis, leading to the formation of dextrins and pregelatinization. Chemical modifications involve changes through covalent linkages. Specifically, these include cross-linking or oxidation and introduction of side groups (FAO, 1998). Genetic modifications involve changes in the composition of amylose and amylopectin (Eliasson and Gudmundsson, 1996). Modification of starch using tools of biotechnology is gaining importance worldwide. Today, carbohydrate scientists, using advanced analytical technology and biotechnology for modifying starches both *in vivo* and *in vitro*, are gaining insight into the fine structure of starch molecules. Ultimately, these modifications have resulted in improved functional properties such as reduced viscosity, improved gel stability, appearance, texture, mouthfeel, and resistance to heat (FAO, 1998). Modified starches as green emulsifiers (fat replacers) is another growing area of interest. Some modified starches may be partially resistant to digestion in the small intestine, thereby contributing to RS. Soluble and insoluble starches play many important roles in the human intestine.

8.6.5.5 Dietary Fiber

Fiber is a nonstarch polysaccharide (NSP) derived from plants. The term dietary fiber was originally described as “that portion of food which is derived from cellular walls of plants which is digested very poorly by human beings” (Trowell, 1972). This description of dietary fiber had more to do with a physiological concept rather than any mention of a food carbohydrate. Nevertheless, the implication of dietary fiber in health and disease was made based on the etiology of a number of diseases seen in the West (Burkitt and Trowell, 1975).

The components of dietary fiber are derived mainly from the cell walls of plant material in the diet. These include cellulose, hemicellulose (β -glucans, arabinoxylans, etc.), and pectin, collectively referred to as the NSPs. Food gums, also called hydrocolloids, are polysaccharides and are considered dietary fiber. Lignin, a non-carbohydrate component of the cell wall and a non-polysaccharide, is also included as fiber that is very tough. Most edible fruits and vegetables, with the exception of carrots, contain low levels of lignin.

Dietary fiber is classified as *soluble fiber* (gums/hydrocolloids, pectins) or *insoluble fiber* (cellulose, hemicellulose). Dietary fiber is neither broken down completely nor absorbed. This is because there is no digestive enzyme capable of hydrolyzing the dietary polysaccharide containing glucose units. Dietary fiber is not digested in

the human upper intestine, thus contributing directly very little to the nutritional value. Nevertheless, it can provide some energy after fermentation in the colon. It does contribute, however, by primarily modulating the digestion and metabolism of other nutrients. For example, the physiological properties of soluble fiber can be seen in its principal effects on glucose and lipid absorption from the small intestine, whereas those of insoluble fiber can be seen in its pronounced effects on bowel behavior.

8.6.6 CARBOHYDRATES IN FUNCTIONAL FOODS

The foregoing discussion took a look at food carbohydrates in general. Here, we will focus on the important carbohydrates in functional foods. In addition to being nutritious, these dietary carbohydrates should also be acceptable and functional. Food carbohydrates with important functional properties include complex carbohydrates, RS, and dietary fibers (Cho, Prosky, and Dreher, 1999).

Complex carbohydrates are a large food group and include starch and nonstarch polysaccharides. They have widely differing chemical structures and physiological effects. The nutritional and physiological importance of complex carbohydrates in foods is increasingly being recognized by both consumers and scientists (Agus et al., 2000; Axelsen et al., 2000; Brown, 1996; Cho, Prosky, and Dreher, 1999; Daly et al., 2000; Liu et al., 2000; Meyer et al., 2000; Ohr, 1999; Parks and Hellerstein, 2000; Smith et al., 2000; Thompson et al., 2000). Dietary fibers as food carbohydrate have been studied for their functional properties (Jenkins et al., 2000; Lu et al., 2000; Ohr, 1999; Walqvist, 1994). The functional properties of oligosaccharides with beneficial health effects have been studied (Ohr, 1999; Oku, 1996). Oligosaccharides, as prebiotics in the maintenance of gut health, have been investigated (Coussement, 1997; Ohr, 1999). Inulin derived from chicory (Roberfroid, 2000; Sheehy and Morrissey, 1998), fructooligosaccharides derived from inulin (Coussement, 1997; Lu et al., 2000; Ohr, 1999; Roberfroid, 1996), and human milk oligosaccharides (Engfer, 2000) have also been investigated for their functional properties with beneficial health effects.

8.6.7 ANALYTICAL ASPECTS OF FUNCTIONAL FOODS

Like all biochemical sciences, advances in food and nutrition sciences can be empowered by advances in analytical sciences. The analysis of foods in general and functional foods in particular is not easy because the food matrix is a multicomponent system composed of carbohydrates, amino acids, proteins, lipids, flavonoids, flavor, aroma, and taste compounds. Food analysis remains the absolute that determines, adds to, and advances the knowledge of nutrition science as an evolving process. It is therefore important to establish the necessary analytical methodologies with standard reference materials. Once the proper food analyses are performed, this analytical information must then be presented in a manner that is both useful and informative to the consuming public. The importance of food analysis and the information gained from such analytical studies are being recognized by institutes such as the National Institutes of Health (NIH), the FDA, the USDA, and the National Institute of Standards and Technology (NIST) (DuPont, 1999).

Spectroscopy was the early technique and tool of choice in food analysis. Specifically, near-infrared (NIR) spectroscopy was used with many applications for the rapid analysis of foods. NIR was used in the systematic analysis of food components such as moisture, protein, and fat, but also sugars and organic acids. The advantages of this technique were its simplicity and versatility. It is still used along with other spectroscopic techniques such as Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopy.

One of the challenges in the area of functional foods remains the analysis of the particular food component(s), its physicochemical properties and their role in different applications, and ultimately its relevance to nutrition. Efforts to establish analytical methods using standard extracts for botanicals have been the subject of a number of presentations offered at the Texas A & M University (College Station, TX) short course on Nutraceuticals and Functional Foods (Mannie, 2000). The identification of food components present in a food sample can be rapidly established by chromatographic methods. Chromatography as a tool and technique is rapidly gaining importance in food analysis. Commercial growth and success have led to increased availability and reliability of analytical techniques and tools. Current state-of-the-art technology includes chromatographic methods such as HPLC and gas chromatography (GC). Both HPLC and GC, independently and in tandem with mass spectrometry (MS), combine to make them the most powerful analytical techniques available to the food scientist.

HPLC is emerging as the technique of choice for the routine analysis of functional foods. This can be seen in HPLC applications to botanicals and herbals (Mannie, 2000). HPLC methods are being validated and promoted by several organizations involved in the analysis of functional foods. For example, the Institute for Nutraceutical Advancement's Methods Validation Program (INAMVP), an industry-wide consortium, selects and validates methods for quantifying compounds used in dietary supplements. The U.S. Pharmacopoeia (USP) has created an independent herbal division that will offer methods of analysis for bulk herbs, standardized herbal extracts, and finished herbal products which will be published in the USP National Formulary. Efforts are underway at the American Herbal Pharmacopoeia (AHP) to publish complete monographs on HPLC protocols for a variety of herbal products. The FDA has designed a clear framework for labeling, allowable structure/function claims, and scientific substantiation for dietary supplement products.

One of the challenges in these analyses is to scientifically substantiate the amount and efficacy of the active ingredient(s) in functional foods. Standardized testing for botanical products is an ongoing effort in the industry today. Establishing standardized marker compounds aids in the quantitative assessment of the quality of the herbal extract. Even though GC and spectrophotometry have been used in the analysis of herbal products, HPLC, with inherent features such as speed, precision, and specificity, is the current method of choice (Ohr, 1999).

HPLC has also been used to analyze herbal tonic products to ensure their therapeutic potency. HPLC profiles of raw botanical materials have been developed with active standards. Testing the finished herbal product is a real challenge. The herbal food matrix is a complex, consisting of not only herbs, but also other components such as flavor compounds, grains, and other ingredients. Therefore, isolating

the herbal components is not an option. On the other hand, the raw herbal material is routinely analyzed to meet the standard specified by the HPLC profile(s) as quality assurance.

8.6.8 ANALYTICAL ASPECTS OF CARBOHYDRATES IN FUNCTIONAL FOODS

The nutritional value of a dietary food carbohydrate depends, to a large extent, on the amount and type of carbohydrate that is provided to elicit a function. This underlying concept of nutrition forms the basis for all analytical methods used to determine the carbohydrate nutritive value of a food. One of the most commonly performed analysis is the determination of carbohydrates. As mentioned above, these carbohydrates include simple sugars, sugar alcohols, oligosaccharides, and polysaccharides.

HPLC, GC, and enzymatic methods have been traditionally used for the specific analysis of monosaccharides, disaccharides and sugar alcohols (polyols) for some time now (Asp, 1995; Greenfield and Southgate, 1992; Southgate, 1991). HPLC along with RID and GC have been the techniques of choice for the simultaneous determination of different monosaccharides. The enzymatic methods, while being very specific, require highly purified enzymes for the determination of specific individual sugars in a mixture without the need for a large investment in analytical instrumentation. This can be seen in the analysis of a single sugar such as glucose as the end point in the analysis of starch. While there are available HPLC methods for the determination of sugar alcohols or polyols, they have usually been determined by GC methods as alditol acetate derivatives.

HPLC and GC have been the techniques of choice for analyzing oligosaccharides. These techniques have worked well for small oligosaccharides or purified preparations. However, these techniques have not been successful for the analysis of oligosaccharides in complex foods. Here, the carbohydrates in complex foods have been initially hydrolyzed using enzymes to free the monosaccharides, followed by their determination using HPLC. For example, the maltooligosaccharides have to be extracted prior to their analysis of starch. Otherwise, they are recovered as starch.

Oligosaccharides, by definition, comprise anywhere from three to nine monosaccharide units. On the other hand, polysaccharides contain ten or more monosaccharide units. From an analysis standpoint, oligosaccharides can be separated from polysaccharides based on their solubility in 80% (v/v) aqueous ethanol. This alcohol solubility of carbohydrates is dependent not only on their degree of polymerization (DP), but on their molecular structure as well. For example, a highly branched complex carbohydrate may exhibit greater solubility in 80% ethanol despite a considerably higher degree of polymerization ($DP > 10$). Nevertheless, analytically speaking, the separation of oligosaccharides from polysaccharides is one that cannot be based on their DP (Asp et al., 1992).

Starch in foods has been analyzed mainly by enzymatic hydrolysis to free the glucose, followed by its specific determination. From a nutritional standpoint, starch is present in foods as digestible and resistant starch. RS is that which is not absorbed in the small intestine of healthy humans. From an analytical standpoint, the distinction between digestible and resistant is unclear (Björk, 1996). Nevertheless, RS is

virtually insoluble in water, and techniques for analyzing total starch in foods have employed either an alkaline [potassium hydroxide (KOH)] or organic solvent [dimethyl sulfoxide (DMSO)] treatment to successfully disperse the crystalline starch fractions and keep them solubilized. Quantitative analysis of RS in foods has utilized enzymes for their determination (Björk, 1996). Nevertheless, techniques for analyzing RS in foods need to be established and tested in formal collaborative studies.

NSPs have been determined using a combination of procedures. Starch is first solubilized and subjected to enzyme hydrolysis. After removal of low molecular weight carbohydrates, including starch hydrolysis products, the NSPs are hydrolyzed to release their monomeric components followed by their quantitative analysis. The critical step is the acid hydrolysis of NSP that must be optimized to achieve complete hydrolysis without degradation of the free monomers (Cho, Prosky, and Dreher, 1999; FAO, 1998). The free monomeric units obtained from NSP have specifically been analyzed as alditol acetate derivatives using GC. The preferred method for analyzing uronic acids derived from pectic substances remains the colorimetric method. There is also a colorimetric method available for determination of total NSPs. NSPs can be fractionated into cellulose and noncellulose polysaccharides using a combination of sequential extraction and hydrolysis. For example, cellulose can be first treated with concentrated acid to disperse the polymer, followed by hydrolysis in dilute sulfuric acid. HPLC analysis of NSPs has been gaining in popularity.

Dietary fiber in foods has been analyzed by three different methods. These three methods have been subjected to comprehensive testing in formal collaborative studies, receiving approval as official methods from the Association of Official Analytical Chemists (AOAC) and the Bureau Communautaire de Reference (BCR) of the European Community (Cho, Prosky, and Dreher, 1999; FAO, 1998). Briefly, the three methods are described below.

The first methods are the AOAC methods which contain the enzymatic-gravimetric methods of Prosky et al. and Lee et al. These methods were established from procedures directed at simulating the digestion in the small intestine of humans to obtain an undigested residue as a measure of dietary fiber. This residue is then corrected for any associated protein and ash. In the absence of KOH or DMSO, starch that resists the enzyme amylase in the assay remains as the fiber component. The sample is milled and subjected to enzymatic hydrolysis using a heat-stable amylase at 100°C. Two of the principal forms of RS, RS1 (physically enclosed starch within intact cell structures) and RS2 (raw starch granules), will not be included. RS3 (retrograded amylose) is included and is the principal form of RS in processed foods. Lignin, a noncarbohydrate component of the cell wall and a non-polysaccharide, is also included along with some tannins. These components comprise a very small proportion of most foods, but can be found in substantial amounts in some unconventional raw materials or special fiber preparations (Cho, Prosky, and Dreher, 1999; FAO, 1998).

The second methods are the enzymatic-chemical methods of Englyst et al. These methods measure the NSP specifically, either as total NSP (reducing substances) using the colorimetric method or as individual monomeric components using techniques such as GC or HPLC. DMSO is used in the initial step to ensure complete removal of starch,

and lignin is not analyzed. The difference between the AOAC methods and these methods is mainly due to RS and lignin (Cho, Prosky, and Dreher, 1999; FAO, 1998).

The third method is the enzymatic-chemical method of Theander et al., also known as the Uppsala method. The hydrolysis conditions and monomeric determination using GC bear a close resemblance to the Englyst method. The difference is that the DMSO step is eliminated and a gravimetric estimate of lignin is added to obtain the dietary fiber. Results obtained using this method and the AOAC methods are in close agreement (Cho, Prosky, and Dreher, 1999; FAO, 1998).

8.6.9 HPAEC-PAD OF CARBOHYDRATES IN FUNCTIONAL FOODS

As mentioned in the foregoing section, carbohydrates in foods and functional foods can be analyzed using the techniques of HPLC or GC. HPLC techniques utilizing sensitive ED are becoming increasingly popular compared to GC for their direct detection of carbohydrates with high sensitivity and specificity and without the need for sample derivatization. Specifically, the technique of HPAEC-PAD is fast becoming the technique of choice. HPLC techniques with RID are greatly limited in their ability to analyze complex carbohydrates in foods.

HPAEC-PAD is now well established for the direct detection of monosaccharides, disaccharides, oligosaccharides, polysaccharides, and sugar alcohols. This technique is proving to be more than capable of analyzing the various components obtained from the hydrolysis of complex carbohydrates and dietary fiber. The technique of HPAEC-PAD has recently been reviewed along with its application to the analysis of the components derived from the hydrolysis of complex carbohydrates and dietary fiber (Henshall, 1999).

Human milk oligosaccharides in one study were analyzed by HPAEC-PED using stachyose as the internal standard (Engfer et al., 2000).

Oligosaccharides produced by the action of an enzyme, lichenase [(1,3)(1,4)- β -D-glucan-4-glucanhydrolase, E.C. 3.2.1.73], on oat β -glucans were analyzed by HPAEC-PAD. These oligosaccharides obtained from lichenase-digested oat β -glucans were identified as tri- and tetrasaccharides (Weisz et al., 1999).

Maltodextrins, dextrans, inulin, and other oligosaccharides have been analyzed by HPAEC-PAD (Dionex Corp.).

8.6.9.1 Food Additives and Nutriment

Ginseng saponins are the major components of ginseng, which is gaining importance as an herbal medicine. Park et al. (1994) determined ginseng saponins from ginseng root to compare the performance of HPAEC-PAD with HPLC-UV. With the PAD system, nanogram-level detection limits were achieved which were two orders of magnitude lower than HPLC-UV analysis.

As discussed previously, carbohydrate analysis of foods is an effective means of identifying cases of adulteration. Low (1996) describes several projects in which adulteration was easily detected using the carbohydrate profile from both the pure food and the sweeteners used in the adulteration process. Grapefruit juice and honey adulteration can be determined by comparing the fingerprint obtained for these

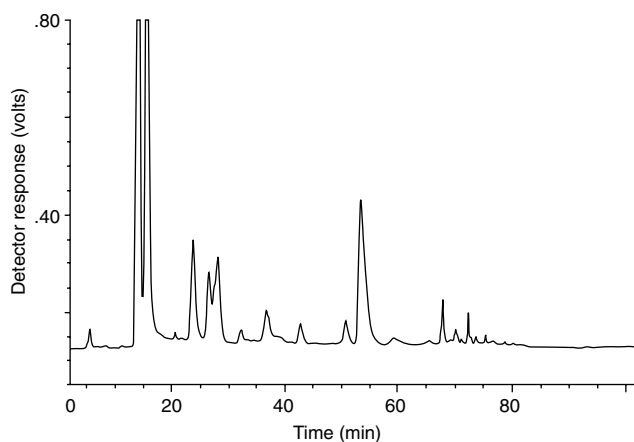


FIGURE 8.9 Chromatogram of the oligosaccharide profile of a pure honey sample. Chromatographic conditions: two CarboPac PA1 columns (Dionex) connected in series; gradient elution was used with NaOH as the primary eluent. Postcolumn addition of NaOH was used to minimize baseline drift. PAD at a gold electrode was used. (Reprinted with permission from Swallow, K.W. and Low, N.H., *J. Assoc. Off. Anal. Chem.*, 77(3), 695–702, 1994.)

products using HPAEC-PAD with the fingerprint obtained for sweeteners such as high-fructose corn syrup. Using these profiles, adulteration can be easily identified.

Analysis of honey for carbohydrate content is a challenging problem. Glucose and fructose are the major components, but, in addition, honey contains at least 11 disaccharides, 11 trisaccharides, and other higher oligosaccharides. Swallow and Low (1990) analyzed honey from different sources to evaluate the applicability of HPAEC-PAD for this analysis. Glucose and fructose were determined by simply diluting the honey with water and analyzing by HPAEC-PAD. Analysis of the purified oligosaccharide samples revealed a unique fingerprint for honeys from different natural sources. Figure 8.9 shows a chromatogram of the carbohydrate profile obtained for a pure honey. Fingerprints of this type can be used to detect adulteration of honey with inexpensive sweeteners. In a later publication, Swallow and Low (1994) demonstrated the use of HPAEC-PAD for the determination of honey adulteration. In this work, samples from 44 different honey sources were analyzed as above (Swallow and Low, 1990), and, although the oligosaccharide profiles were similar, a unique fingerprint was discerned for each one. The sources of the variation in the honey are numerous, ranging from the botanical source to the storage temperature. Honey samples were then purposely adulterated with high-fructose corn syrup and invert syrup to determine changes in the profile. In both cases, the presence of the adulterating substance was easily detected. Determination of the adulteration of maple syrup by HPAEC-PAD was also shown by Stuckel and Low (1995).

Monosaccharides and disaccharides in molasses were determined by HPAEC-PAD, which is the International Commission for Uniform Methods of Sugar Analysis official method (Dionex Technical Manual, 1995). This was an interlaboratory study

which involved 11 laboratories. The reproducibility of this method was excellent for the different laboratories and the results agreed with a GC method.

Larew and Johnson (1988) quantified the percentage of maltooligosaccharides found in corn syrup using HPAEC-PAD. This work is distinguished from other corn syrup determinations because they added a glucoamylase reactor between the separation and detection steps. The enzyme reactor served to increase the sensitivity for maltooligosaccharides, and, since the analytes are converted to glucose during the enzyme reaction, only a glucose calibration curve is required for calibration. This system worked well for the corn syrup samples, and it did decrease the work required in the quantitation step. Stefansson and Lu (1993) also determined maltooligomers in corn syrup directly using reversed-phase ion pair chromatography-PAD to determine the compatibility of the ion pair chromatography with PAD. This system proved compatible with PAD and more versatile than ion-exchange chromatography because the ion pair agent could be changed to affect different chromatographic selectivity.

8.6.9.2 Solid Foods

HPAEC-PAD is applicable for complex samples. One of the earliest studies using HPAEC-PAD showed the analysis of high-fructose corn syrup and potato chips (Edwards and Haak, 1983). The corn syrup assay was of immediate interest to manufacturers, because the presence of higher saccharides affects the taste of the syrup by imparting a bitter taste. The use of HPAEC-PAD for quality control of the product was demonstrated with the analysis of glucose, sucrose, and lactose to determine the level of seasoning present in the finished product. Rocklin and Pohl (1983) published a more detailed study of determining glucose, fructose, and lactose from extracts of flavored potato chips in the presence of high concentrations of salt.

Van Riel and Olieman (1991) took advantage of the sensitivity of HPAEC-PAD in their sample preparation of dairy products for mono- and oligosaccharide analysis using ultrafiltration. For dairy products containing 0.5% individual saccharides, 1 g was diluted to 100 ml, and 2 ml of the resulting solution was filtered. Recovery of galactose, fructose, saccharose, lactulose, glucose, maltose, maltotriose, and maltopentaose was determined by spiking 50 mg of each into 10 g of skim milk. Recoveries ranged from 98.2% for maltopentaose to 101.8% for saccharose. Repeatabilities were less than 5.3%. Sample matrices included fruit yogurt, candy, and infant formula. Figure 8.10 shows the HPAEC-PAD traces for each of these samples.

Common sugars, such as glucose, fructose, and sucrose, in a wide variety of foods can be determined after dilution and filtration prior to HPAEC-PAD analysis. Such foods include tomato ketchup, butterscotch candy, and chocolate (Dionex Technical Manual, 1995). Sample preparation for the high-fat chocolate includes supercritical fluid extraction to remove the fat. Impurities in sweeteners can be detected, such as glucose and fructose in sucrose (Dionex Technical Manual, 1995).

Artificial sweeteners include sucrose derivatives as well as sugar alcohols. Sucralose is a sweetener which is 400 to 800 times as sweet as sucrose. It is a selectively chlorinated sucrose. Ichiki and co-workers determined sucralose in foods (1996), and trace impurities 4-Cl-galactose and 1,6-Cl-fructose were detected in a

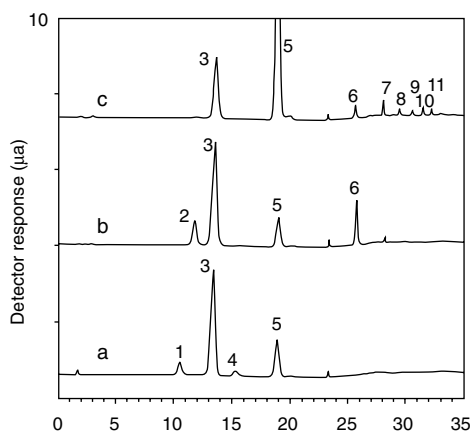


FIGURE 8.10 Chromatograms of saccharides in (a) fruit yogurt, (b) candy, and (c) infant formula. Peak identification: 1, D-galactose; 2, D-glucose; 3, saccharose; 4, D-fructose; 5, lactose; 6, maltose; 7, maltotriose; 8, maltotetraose; 9, maltopentaose; 10, maltohexaose; and 11, maltoheptaose. Conditions: linear gradient from 0 to 0.25 M sodium acetate in 0.1 M sodium hydroxide on CarboPAC PA1 column (Dionex). Detection: PAD. (Reprinted from Van Riel, J. and Olieman, C., *Carbohydr. Res.*, 215, 39–46, 1991. With kind permission from Elsevier Science.)

sucralose sample (Dionex Technical Manual, 1995). Other sugar substitutes such as kestose, maltrin, and inulin are also separated and detected using HPAEC-PAD (Dionex Technical Manual, 1995). Sugar alcohols including glycerol, sorbitol, and mannitol have been determined in hard candies and chewing gum (Dionex Technical Manual, 1995). Sample preparation involves either dissolution or sonication, followed by dilution.

The determination of lactose, galactose, and dextrose in grated cheeses was improved by using anion-exchange chromatography with pulsed amperometric detection (Pollman, 1989). Previous LC methods suffered from a lack of sensitivity, failure to resolve galactose and dextrose, and interference from the presence of salt in the cheese in the determination of dextrose.

Monosaccharide analysis following acid hydrolysis is a common use of HPAEC-PAD. Garleb, Bourquin, and Fahey (1989, 1991) found that HPAEC-PAD technology was superior to previous HPLC techniques and that the hydrolysis method was the limiting factor in neutral monosaccharide analysis. Their aim was to compare three hydrolytic methods, one using trifluoroacetic acid (TFA) and two using sulfuric acid (H_2SO_4) under different temperature and time parameters. The substrates hydrolyzed included xylan, microcrystalline cellulose, wheat bran, apples, wheat straw, and alfalfa. Monosaccharides quantified were arabinose, xylose, glucose, galactose, mannose, and rhamnose. They found that using TFA for hydrolysis led to less monosaccharide degradation, but that the procedures using H_2SO_4 yielded greater recovery of monosaccharides. Also, the temperature, rather than duration of hydrolysis, yielded higher recoveries of monosaccharides for certain substrates.

Quigley and Englyst (1992) analyzed NSPs, which are measured as an index of dietary fiber. Their analysis required enzymatic release of starch followed by its acid hydrolysis. Neutral and amino sugars are released using 12 *M* sulfuric acid at 35°C for 1 h followed by 2 *M* sulfuric acid at 100°C for 1 h. Sample preparation following hydrolysis consisted of addition of an internal standard for both neutral and amino sugars and addition of NaOH for amino sugars to adjust the pH. Removal of sulfate ions is accomplished on-line using a precolumn guard and column switching. Samples hydrolyzed and analyzed for neutral sugars were guar gum, mucin, chin and cellulose, and the NSP residue from haricot bean, oat bran, mycoprotein, wheat bran, soya bran, wholemeal flour, carrot, cabbage, garden pea, sugar beet, mushroom, and ileostomy effluent. The other components of NSP are uronic acids. Quigley and Englyst (1994) released uronic acids from pectin, in which values for uronic acids are known, in order to understand the time course of release. These values were used to determine hydrolysis, either by acid or enzymes, times for release of uronic acids from NSP from food sources. The samples analyzed include linseed, carrot, potato, haricot bean, brussels sprout, cabbage, parsnip, and wholemeal bread. Determination of glycouronic acids by HPAEC-PAD was also accomplished by Martens and Frankenberger (1990a, b). Samples of orange peel, acacia powder, straw, animal waste, and soil were acid hydrolyzed followed by enzyme catalysis to extract the galacturoic and glucuronic acids. The HPAEC-PAD method was superior to an existing HPLC-UV method because of the sensitivity and precision achievable.

Pectin is an important natural polymer because it is responsible for determining cell wall strength and flexibility. Oligogalacturonic acids are released from polygalacturonic acid in pectin and are regulators of physiological responses in plants. Hotchkiss and co-workers (1990, 1993) reported on the analysis for oligogalacturonic acids in plant tissues by HPAEC-PAD. HPAEC-PAD analysis yielded a direct method in which a complete oligosaccharide profile was obtained. In a later study, Hotchkiss and co-workers (1996) investigated the pectin structure in peaches to gain insight into the ripening process. Peach tissues were extracted and enzyme digested with endopolygalacturonase with analysis performed at 0, 15, and 45 min to observe the progression of the enzymatic digestion. Results were compared with a high-performance size exclusion analysis to provide a more complete picture of the peach ripening process.

Galacturonate in pectin was analyzed by HPAEC-PAD and compared to an existing colorimetric procedure (Garaleb, Bourquin, and Fahey, 1991). Samples of apple, cucumber, celery, grapefruit, and radish were lyophilized and the pectin was isolated from the total dietary fiber fraction. No statistically significant difference between the HPAEC-PAD and colorimetric method was detected; however, the colorimetric procedure could not differentiate between glucuronate and galacturonate. In addition, the HPAEC-PAD procedure was used to determine the chemical composition of the pectin isolated from the samples. In this case, the HPAEC-PAD method was superior to a gas-liquid chromatography method primarily because PAD eliminates the need for derivitization.

During times of stress, plants will accumulate low molecular weight metabolites within their tissues. Adams et al. (1993) analyzed tissue from several types of plants for evidence of stress through the presence of sugar alcohols, specifically inositol. Samples of flower petals were extracted with a liquid-liquid extraction, desalted,

vacuum dried, reconstituted in water, passed through a polystyrene-divinylbenzene SPE cartridge, and analyzed by HPAEC-PAD. HPAEC-PAD proved to be excellent for the complex samples which resulted from the extraction process with no discernable interferences for the peaks of interest.

A specialty pectin, which is used as a fat substitute, is a partially methylated polygalacturonic acid. Pectins from different sources have different oligogalacturonic acid profiles. Separation of pectins of various degrees of polymerization is possible using anion-exchange chromatography with an acetate gradient (Dionex Technical Manual, 1995).

Koizumi and co-workers (1989) used HPAEC-PAD to determine D-glucooligosaccharides and D-glucopolysaccharides with DP > 50. They also determined that sensitivity is not independent of molecular weight, rather it increases for each HCOH group present, which makes PAD a good detection choice for oligosaccharides. In addition, Koizumi and co-workers demonstrated the separation of depolymerization products of the amylopectin portion of starch from different crops including rice, corn, sweet potato, and edible canna. Baseline separation of amylopectins from a DP of 6 to 60 is accomplished in 40 min (Koizumi, Fukuda, and Hizukuri, 1991). Under gradient conditions, acetate ion is typically the preferred "pusher" ion, in that it offers rapid equilibration, is PAD inactive, and allows for near maximal resolution of carbohydrate moieties. Recently, Wong and Jane (1995) compared the effects of acetate and nitrate as pushing agents in HPAEC-PAD for the separation and detection of debranched amylopectin. They found that in comparison with the commonly used pushing agent, nitrate offered greater reproducibility, accuracy, and lower limits of detection. Figure 8.11 shows the HPAEC-PAD chromatographic profiles of the enzymatically debranched tapioca and wheat amylopectins using a nitrate gradient. Impressively, baseline resolution of peaks up to a DP of 66 was achieved. The separations were designed to be completed within 100 min.

Slaughter and Livingston (1949) used HPAEC-PAD to analyze cereal grasses for fructan isomers. They found that the HPAEC-PAD method was far superior to existing methods of analysis for isomeric fructans, which were a combination of reverse- and normal-phase separation. The HPAEC-PAD method was complete within 20 min. Resolution of closely related oligosaccharides clearly highlights the power of high-pH anion-exchange chromatography, especially when combined with direct detection via PAD.

The determination of trace levels of glycosides in grape musts was undertaken by Pastore, Lavagnini, and Versini (1993). They hydrolyzed the glycosides and analyzed the monosaccharides derived, thus circumventing analysis of the great variety of aglyconic constituents. Analysis of the monosaccharides included separation on a CarboPAC PA1 column using 20 mM NaOH at 15°C. Regeneration of the column using 0.4 M acetic acid was performed daily. Decreasing retention times and increasing peak heights were accounted for by frequently injecting standards. Detection using PAD took place at a gold microband electrode fabricated by the authors. The authors determined that the microband electrode did not exhibit lower limits of detection, but that stability of the baseline was more rapidly attained. Reproducibility for the microband electrode was 3% for 10 repeated injections of 20 μ l of 2 mg/l glucose. The sugars detected in the grape must included rhamnose, arabinose, glucose,

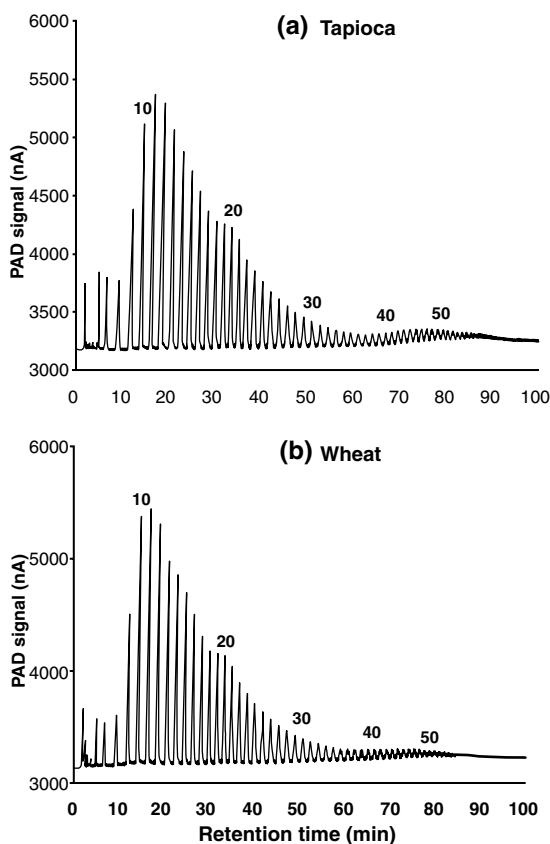


FIGURE 8.11 Chromatographic profiles of the enzymatically debranched (a) tapioca amylopectin and (b) wheat amylopectin using HPAEC-PAD. Peak numbers indicate the degree of polymerization. (Reprinted with permission from Wong, K.S. and Jane, J., *J. Liq. Chromatogr.*, 18, 63, 1995. Courtesy of Marcel Dekker.)

xylose, fructose, and apiose. The fructose is conjectured to come from the must and not from a hydrolyzed glycoside. This is the first report of xylose as a component of grape must glycosides.

Frias and co-workers (1996) undertook the analysis of the raffinose family of oligosaccharides (RFO). Determination of RFO is important because these compounds have been associated with flatulence in animals and humans and have therefore limited the consumption of foods with a high RFO content. Extraction of RFO from pea seed flour is accomplished using 80% ethanol, and the solution is further purified using C_{18} SPE cartridges. Analysis of the eluent was performed using CZE-UV₂₂₀ and HPAEC-PAD. Sugars quantitated were sucrose, raffinose, stachyose, and verbascose. Lactose was used as an internal standard for capillary zone electrophoresis (CZE). Results were similar using each detection scheme, although the HPAEC-PAD was ca. 1000 times more sensitive than the CZE-UV.

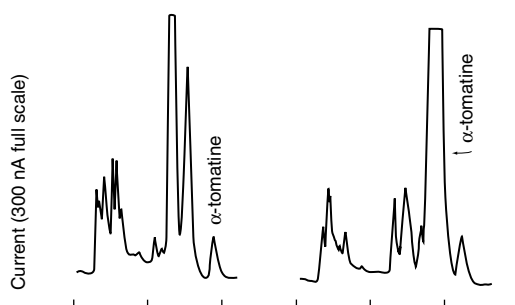


FIGURE 8.12 HPLC chromatograms of α -tomatine in field-grown (left) ripe red tomatoes and (right) unripe green tomatoes. Conditions: column, Supelcosil LC-ABZ; mobile phase 25% ACN, 15% MeOH, 100 mM sodium phosphate (monobasic) pH 3. (Reprinted from Friedman, M., Levin, C.E., and McDonald, G.M., *J. Agric. Food Chem.*, 42, 1959–1964, 1994. Copyright 1994 American Chemical Society.)

α -Tomatine in various types of tomatoes was determined using HPLC-PAD by Friedman, Levin, and McDonald (1994). α -Tomatine is a glycoalkaloid found in ripening tomatoes. It is reported to be toxic and have antifungal activity, and its content in tomatoes decreases as the fruit ripens. Separation of glycoalkaloids extracted from fresh tomato was accomplished using reversed-phase chromatography with phosphate/citrate buffer, pH 3.0/ACN/MeOH, followed by PAD at a gold electrode at a detection potential of 600 mV. Figure 8.12 shows chromatograms of red (left) and unripe green (right) field-grown tomatoes. The α -tomatine in the red ripe tomato is much less than in the unripe green tomato, as expected.

Using the extraction procedure and HPLC-PAD as described in their 1994 article, Friedman and Levin (1995) determined the α -tomatine in different parts of the tomato plant as well as in various tomato-base foods. Ripe, red tomatoes contained the least α -tomatine. The content of α -tomatine was greater in unripe green tomatoes and greatest in leaves, small stems, and flowers of the tomato plant. The authors conjecture that the presence of α -tomatine helps to protect against insects and fungal pathogens. They found no α -tomatine in eggplants, as predicted, but did find small amounts in tomatillos, which are not closely related to tomatoes. The authors were able to quantitate the amount of α -tomatine in prepared tomato products such as stewed red tomatoes, pickled green tomatoes, microwaved green tomatoes, and fried green tomatoes.

8.7 REVIEW OF AGRICULTURAL APPLICATIONS

The classification of tobacco products as cigarette or cigar by the Bureau of Alcohol, Tobacco and Firearms is critical for the purpose of application of the correct taxation rate. Since the monosaccharide profile of cigarette and cigar tobacco differs because of the differences in processing, Zook et al. (1996) present a method in which the monosaccharide profile is elucidated and used for this purpose. Samples were prepared by extraction with water, C_{18} SPE cleanup, and HPAEC-PAD analysis. Using

this method, cigarettes are easily differentiated from cigars because cigarettes contain three to ten times more glucose and fructose and the cigars tested lacked sucrose in all cases.

Fructan is known to accumulate in the tissues of temperate plants during times of cool weather. Since the analysis for fructan is difficult, Chatterton et al. (1989) present a method for the analysis of the synthetic intermediate, fructosylsucrose. Purified sample extracts were analyzed by HPAEC-PAD with a limit of detection of 1 mg/ml.

Wilson, Cataldo, and Andersen (1995) studied stress indicators to determine stress in trees. In this work, the presence of sugar alcohols and saccharides was monitored. Samples were prepared by liquid-liquid extraction, followed by drying, reconstitution in water, and analysis by HPAEC-PAD. The PAD procedure was compared with an existing colormetric assay and an HPLC-RID method. The PAD was superior to both methods. Starch was also analyzed by first extracting from the tissue with base and the extract was digested with amyloglucosidase. The resulting samples were analyzed by HPAEC-PAD for glucose. The analysis took only 4 min, so large numbers of samples can be reliably and quickly analyzed with the presented method.

Glucooligosaccharides and polysaccharides were analyzed by hydrophilic interaction chromatography-PAD to evaluate the technique for investigation of polysaccharide structure. Feste and Khan (1992) were successful in fractionating glucooligosaccharides with a run time of 26 min. Oligosaccharide structure was also the focus of work done by Ammeraal and co-workers (1991). In this case, the authors were using HPAEC-PAD to determine the structure of linear and branched glucose oligosaccharides. The authors were successful in separating oligosaccharide chains with two or three branches isomers which are singly branched.

8.8 REVIEW OF INDUSTRIAL APPLICATIONS

Sodium carboxymethylcellulose was analyzed by HPAEC-PAD for the purpose of quality control (Kragten, Kamerling, and Vliegthart, 1992). This analyte is important in the food and coatings industry. The samples were prepared by hot acid hydrolysis. Because the HPAEC-PAD method is direct, it has advantages over existing methods which require derivatization.

The paper industry is notorious for discharging toxic chemicals into natural receiving waters. Ozonolysis is being investigated as an alternative to the bleaching process because it would eliminate the use of chlorinated organic compounds currently used for this purpose. However, with current methods of ozonolysis, the ozone modifies the cellulose such that the pulp properties are lost. Van Nifterik et al. (1993) used HPAEC-PAD to study the action of ozone on cellulose in order to minimize the modification process. Samples were ozonated and then passed through a polyvinylpyrrolidone filter cartridge prior to analysis.

Carbohydrate analysis of samples in various stages of the paper-making process is important to the optimization of the process. Breakdown of polysaccharides to monosaccharides is undesirable. To monitor paper processing, Sullivan and Douek (1994) developed an HPAEC-PAD method for determining monosaccharides in wood, pulp, and process liquor samples. This method yielded an easy, fast method of determining monosaccharides in a quite complex matrix. Figure 8.13 shows a

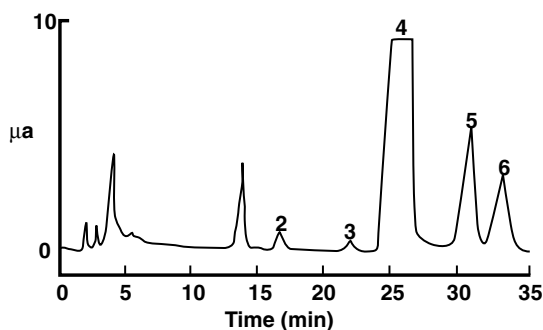


FIGURE 8.13 Chromatogram of an unbleached kraft pulp hydrolyzate. Peaks: 1, 2-deoxy-glucose; 2, arabinose; 3, galactose; 4, glucose; 5, xylose; and 6, mannose. Chromatographic conditions: CarboPac PA-1 + guard (Dionex Corp.); Eluent: 3 mM NaOH; PAD-1 detector (Dionex) with a gold working electrode and Ag/AgCl reference. (Reprinted from Sullivan, J. and Douek, M., *J. Chromatogr.*, 671(1–2), 339–350, 1994. With kind permission from Elsevier Science.)

chromatogram using the HPAEC-PAD method with an SPE cleanup step of an unbleached kraft pulp hydrolyzate. The chromatogram is quite clean with minimal matrix peaks.

Free glycerol content in biofuels is an important indicator of the performance of a particular fuel. Lozano et al. (1996) used HPLC-PAD to determine glycerol in fuel after an extraction of the glycerol with a water–alcohol mixture. The HPLC-PAD method was compared to an established enzymatic method. The HPLC-PAD method compared favorably with the established method because although there was no statistical difference in the results from the two methods, the HPLC-PAD method is easier to perform.

8.9 NONCARBOHYDRATE APPLICATIONS

Several studies have focused on the detection of aliphatic amines by HPLC-PAD (Dobberpuhl and Johnson, 1995; Dobberpuhl, Hoekstra, and Johnson, 1996), but amine detection is oxide-catalyzed and is best performed using IPAD. A fine example involves the use of HPLC-IPAD to directly determine biogenic amines as indicators of seafood spoilage (Draisici et al., 1993). They separated putrescine, histidine, cadaverine, and histamine using a cation-exchange column with an ACN gradient. Figure 8.14 shows the chromatograms of amines extracted from spoiled canned herrings. Detection was performed by IPAD following postcolumn addition of NaOH.

Simple mixtures of amino acids can be separated isocratically using anion-exchange chromatography (Welch et al., 1990). For complex mixtures, it is essential to perform separations with gradient-elution chromatography. Figure 8.15 shows the chromatogram of a protein hydrolyzate containing 17 amino acid residues using HPLC-IPAD with a pH reference electrode (Welch et al., 1989). The amino acid mixture was separated using an anion-exchange column with a quaternary gradient, which incorporated both a pH and an organic modifier gradient. An improved separation of

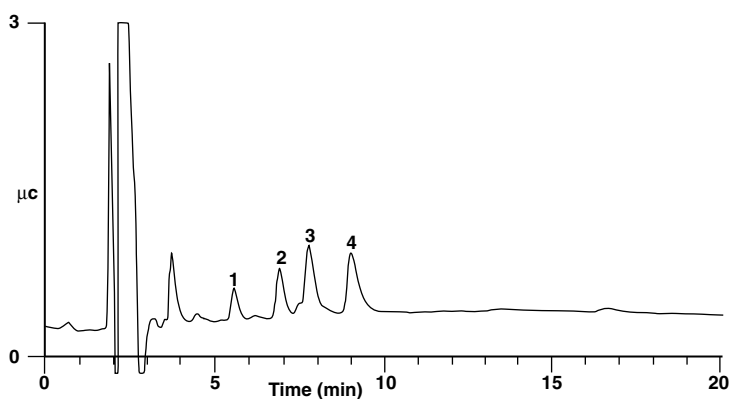


FIGURE 8.14 HPLC-PAD analysis of biogenic amines in fish using HPLC-PAD. Peaks: 1, putrescine; 2, histidine; 3, cadarverine; and 4, histamine. (Reprinted with permission from Draisci, R. et al., *Chromatographia*, 35, 584–590, 1993.)

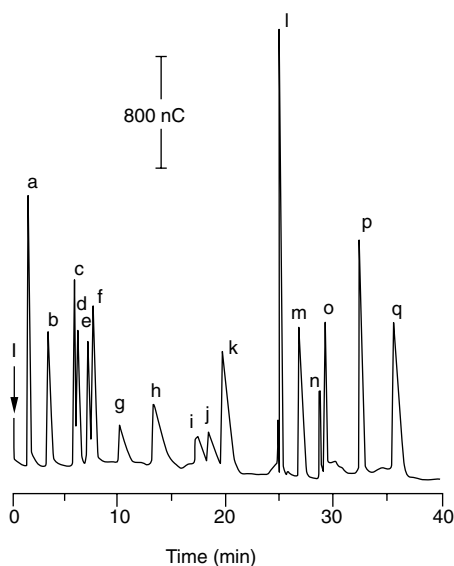


FIGURE 8.15 Separation of a 17-component amino acid hydrolyzate. Conditions: column, Dionex AS-8; gradient, see Welch et al., 1989. Peaks (25 nmole each, except 12.5 nmole for cystine): a, arginine; b, lysine; c, threonine; d, alanine; e, glycine; f, serine; g, valine; h, proline; i, isoleucine; j, leucine; k, methionine; l, histidine; m, phenylalanine; n, glutamic acid; o, aspartic acid; p, cystine; and q, tyrosine. (Reprinted from Welch, L.E., LaCourse, W.R., Mead, D.A., Jr., and Johnson, D.C., *Anal. Chem.*, 61, 555, 1989. Copyright 1989 American Chemical Society.)

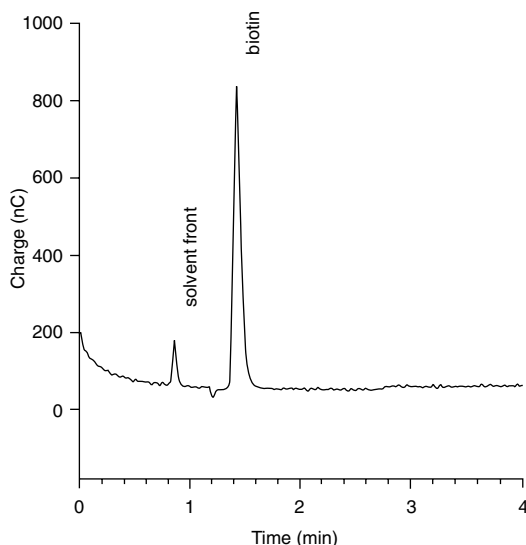


FIGURE 8.16 Chromatogram of biotin (165 pmol injected) using HPLC-IPAD. Conditions: C_{18} 1-mm SepStik column; mobile phase, 90/10 vol% pH 7.2 phosphate buffer, 100 mM ACN; 0.5 μ l injection volume; flow rate, 70 μ l min^{-1} ; and detection, IPAD. (Reprinted from LaCourse, W.R., Dasenbrock, C.O., and Zook, C.M., *Sem. Food Anal.*, 2, 5–41, 1997. With kind permission from Chapman & Hall.)

21 amino acids was performed by Martens and Frankenberger (1992). Presently, detection limits (i.e., $S/N = 3$) for HPLC-IPAD for amino acids are typically 1 to 50 pmole injected with comparable sensitivities for primary and secondary amino acids.

Recently, LaCourse and Owens (1995) have demonstrated the superiority of IPAD over PAD for the determination of thiocompounds using standard reversed-phase conditions. These results were as expected since sulfur detections are Mode II. Figure 8.16 shows the response of biotin (160 pmole injected) using microbore chromatography. Biotin, a thioether, was detected sensitively without derivatization. In addition, IPAD enables the direct determination of thio redox couples [i.e., $-\text{SH}/-\text{S}-\text{S}-$] and numerous other sulfur moieties at a single Au electrode. The high selectivity of PED for thiocompounds under mildly acidic conditions reduces sample preparation and produces simpler chromatograms of complex mixtures. Figure 8.17 shows the determination of thiocompounds in (A) grapefruit juice, (B) watermelon, and (C) chicken liver. In general, the IPAD waveform gives lower limits of detection, more stable baselines, and eliminates oxide-induced artifacts.

More recently, the analysis of milk for sulfur-containing antibiotic residues was performed using a C_8 column with a mobile phase of 20% 500 mM acetate buffer (pH 3.75)–5% ACN–75% water (Dasenbrock, Zook, and LaCourse, 1996). Table 8.4 shows that detection limits of 1 to 5 ppb were achieved with single injections of each compound. All of these detection limits are at or below the target levels for antibiotic residues in milk set by the FDA. The IPAD analysis was also compared with a UV detection scheme by adding a UV detector in line. In all cases, the IPAD detection was more sensitive and selective for the compounds of interest than UV detection.

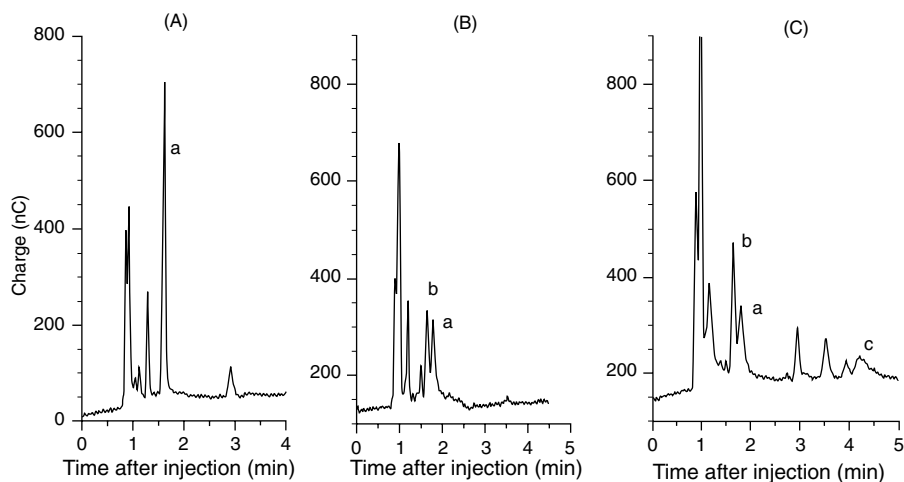


FIGURE 8.17 Detection of thiocompounds in various samples by HPLC-IPAD. Samples were (A) grapefruit juice, (B) watermelon, and (C) chicken liver. Condition: C_{18} 1-mm SepStik column; 99.6% 100 mM phosphate buffer (pH 3)–0.4% acetonitrile; 65 l min^{-1} ; 0.5 l injection volume; and detection, IPAD waveform in Table 8.3. Peaks: a, GSH; b, methionine; and c, GSSG. (Reprinted from LaCourse, W.R. and Owens, G.S., *Anal. Chim. Acta*, 307, 301, 1995. With kind permission from Elsevier Science.)

TABLE 8.4
Summary of the Analytical Figures of Merit
Achievable with HPLC-IPAD

Compound	Target Level (ppb)	LOD ^a (ppb)	LOL ^b (ppb)	CV ^c at 50 ppb
Amoxicillin	10	1	50	1.4%
Ampicillin	10	2	500	1.9%
Cephapirin	20	1	200	1.7%
Penicillin G	5	5	500	3.6%
Cloxacillin	10	5	500	2.4%

Note: These data were collected by injecting each compound separately with a mobile phase chosen to yield similar k' values for each compound.

^a LOD, limit of detection ($S/N = 3$).

^b LOL, limit of linearity.

^c CV, coefficient of variation.

Many of the early efforts to discern the mechanism of anodic oxygen transfer utilized inorganic species as test species. It only follows that these compounds are also applicable to PED following ion chromatography. Wagner and McGarrity (1992) used ion chromatography followed by PAD to determine sulfite in beer. Correlation coefficients of 0.997 or better were obtained with excellent spike recoveries. HPAEC-PAD results were in good agreement with the standard *para*-rosaniline method. In fact, this method was subsequently automated, resulting in excellent precision and accuracy (Wagner, 1995).

8.10 CONCLUSIONS

The significance of PED within chemical and biochemical analysis can be best appreciated in view of the commonly held impression, based on attempted detections at constant (dc) applied potential, that polar aliphatic compounds are generally not electroactive. Furthermore, these compounds often do not possess chromophoric or fluorophoric groups, and, as a consequence, direct and sensitive detection by photometric techniques is not possible. PED has been applied to the *direct*, *sensitive*, and *reproducible* detection of a large variety of polar aliphatic compounds in foods. These compounds include carbohydrates, amines, amino acids, and thiocompounds. This technique is *simple* in that it requires no derivatization.

PED is ideally suited to the detection of virtually all carbohydrates and carbohydrate analogs. In addition, the optimized waveform for any one compound with a class is applicable to the all others in that class. IPAD facilitates Mode II detections and increases gradient compatibility. PED is compatible with all aqueous-based separations, and sulfur-based compounds are selectively detected under typical reversed-phase conditions.

PED offers many advantages over alternate detection schemes for LC and capillary electrophoresis. Because ED relies on reaction at the electrode surface, detector cells can be miniaturized without sacrificing sensitivity. This advantage makes them especially suited for microbore techniques. As microbore techniques gain prominence, low-volume detection schemes will become more important. Pulsed potential cleaning eliminates the need for daily polishing of the electrode which renders PED more convenient experimentally than dc amperometry. PED is sensitive and selective for specific functional groups on the analyte, excluding components which do not contain those functional groups. In this manner, some potential interferents are not detected and the data interpretation is simplified. When analyzing complex sample matrices, such as food matrices, this advantage becomes quite significant (LaCourse, Dasenbrock, and Zook, 1997).

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REFERENCES

- Adams, P., Zegeer, A., Bohnert, H.J., and Jensen, R.G., *Anal. Biochem.*, 214(1), 321, 1993.
- Adams, R.N., *Electrochemistry at Solid Electrodes*, Marcel Dekker, New York, 1969.
- Agus, M.S.D. et al., *Am. J. Clin. Nutr.*, 71, 901, 2000.
- Ammeraal, R.N., Delgado, G.A., Tenbarga, F.L., and Friedman, R.B., *Carbohydr. Res.*, 215(1), 179, 1991.
- Arai, S., *Biosci. Biotechnol. Biochem.*, 60, 9, 1996.
- Armstrong, G., Himsworth, F.R., and Butler, J.A.V., *Proc. R. Soc. London A*, 143, 89, 1934.
- Asp, N.-G., Classification and methodology of food carbohydrates as related to nutritional effects, *Am. J. Clin. Nutr.*, 61(4, Suppl.), 930S, 1995.
- Asp, N.-G., Schweizer, T.F., Southgate, D.A.T., and Theander, O., Dietary fibre analysis, in *Dietary Fibre. A Component of Food. Nutritional Function in Health and Disease*, Schweizer, T.F. and Edwards, C.A., Eds., Springer-Verlag, London, 1992, p. 57.
- Austin, D.S., Polta, J.A., Polta, T.Z., Tang, A.P.C., Cabelka, T.D., and Johnson, D.C., *J. Electroanal. Chem.*, 108, 227, 1984.
- Axelsen, M. et al., *Am. J. Clin. Nutr.*, 71, 1108, 2000.
- Berger, T.A., U.S. Patent No. 4,496,454, Hewlett-Packard Corp., Avondale, PA, 1985.
- Bernarl, J.L., Del Nozal, M.J., Toribio, L., and Del Alamo, M., *J. Agric. Food Chem.*, 44(2), 507, 1996.
- Björk, I., Starch: nutritional aspects, in *Carbohydrates in Food*, Eliasson, A.-C., Ed., Marcel Dekker, New York, 1996, p. 505.
- Breiter, M.W., *Electrochim. Acta*, 8, 973, 1963.
- Brown, I., *Nutr. Rev.*, 54(11, Part II), S115, 1996.
- Burkitt, D.P. and Trowell, H.S., *Refined Carbohydrate Foods and Disease: Some Implications of Dietary Fibre*, Academic Press, London, 1975.
- Chapman, N., *Prepared Foods*, February 2000.
- Chatterton, J.N., Harrison, P.A., Thornley, W.R., and Bennett, J.H., *Plant Physiol. Biochem.*, 27(2), 289, 1989.
- Cheney, S.J., *Vegetarian Times*, June 1995.
- Cho, S.S., Prosky, L., and Dreher, M., Eds., *Complex Carbohydrates in Foods*, Marcel Dekker, New York, 1999.
- Clark, D., Fleishman, M., and Fletcher, D., *J. Electroanal. Chem.*, 36, 137, 1972.
- Clydesdale, F.M., *Nutr. Rev.*, 55, 413, 1997.
- Corradini, C., Cristalli, A., and Corradini, D., *Ital. J. Food Sci.*, 6(1), 103, 1994.
- Corradini, C., Cristalli, A., and Corradini, D., *J. Liq. Chromatogr.*, 16(16), 3471, 1993.
- Coussement, P., Non-digestible oligosaccharides for functional foods, in *Proceedings of the First Vitafoods International Conference*, Blenford, D.E., Ed., Food Tech Europe, Copenhagen, 1997.
- Craig, W.J., *J. Am. Diet. Assoc.*, 97, S199, 1997.
- Daly, M.E. et al., *Am. J. Clin. Nutr.*, 71, 1516, 2000.
- Dasenbrock, C.O., Zook, C.M., and LaCourse, W.R., Analysis of Antibiotics in Milk Using HPLC-IPAD, Paper presented at Ohio Valley Chromatography Symposium, Oxford, OH, June 1996.
- Dionex Corp., Dionex Application Note 67, Dionex Corporation, Sunnyvale, CA.
- Dionex Technical Manual, *Better Solutions for Food and Beverage Analysis*, Dionex Corporation, Sunnyvale, CA, 1995.
- Dobberpuhl, D.A., Hoekstra, J.C., and Johnson, D.C., *Anal. Chim. Acta*, 322, 55, 1996.
- Dobberpuhl, D.A. and Johnson, D.C., *Anal. Chem.*, 67, 1254, 1995.
- Draisci, R., Cavalli, S., Lucentini, L., and Stacchini, A., *Chromatographia*, 35, 9, 1993.

- DuPont, J., *Nutr. Today*, 34(6), 234, 1999.
- Edwards, P. and Haak, K.K., *Am. Lab.*, April, 78, 1983.
- Edwards, W.T., Pohl, C.A., and Rubin, R., *Tappi J.*, 70, 138, 1987.
- Eliasson, A.-C. and Gudmundsson, M., Starch: physicochemical and functional aspects, in *Carbohydrates in Food*, Eliasson, A.-C., Ed., Marcel Dekker, New York, 1996, p. 431.
- Engfer, M.B. et al., *Am. J. Clin. Nutr.*, 71, 1589, 2000.
- Englyst, H.N. and Cummings, J.H., Non-starch polysaccharides (dietary fiber) and resistant starch, in *New Developments in Dietary Fiber*, Furda, I. and Brine, J., Eds., Plenum Press, New York, 1990, p. 205.
- Englyst, H.N., Kingman, S.M., and Cummings, J.H., Classification and measurement of nutritionally important starch fractions, *Eur. J. Clin. Nutr.*, 46, S33, 1992.
- Ewing, A.G., Dayton, M.A., and Wightman, R.M., *Anal. Chem.*, 53, 1842, 1981.
- FAO, Carbohydrates in Nutrition, Report of a Joint FAO/WHO Expert Consultation, *FAO Food and Nutrition Paper 66*, Rome, Italy, 1998.
- Feste, A.S. and Khan, I., *J. Chromatogr.*, 630(1-2), 129, 1992.
- Fleet, B. and Little, C.J., *J. Chromatogr. Sci.*, 12, 747, 1974.
- Frias, J., Price, K.R., Fenwick, G.R., Hedley, C.L., Sorensen, H., and Vidal-Valverde, C., *J. Chromatogr.*, 719, 213, 1996.
- Friedman, M. and Levin, C.E., *J. Agric. Food Chem.*, 43, 1507, 1995.
- Friedman, M., Levin, C.E., and McDonald, G.M., *J. Agric. Food Chem.*, 42, 1959, 1994.
- Garleb, K.A., Bourquin, L.D., and Fahey, G.C., Jr., *J. Agric. Food Chem.*, 37, 1287, 1989.
- Garleb, K.A., Bourquin, L.D., and Fahey, G.C., Jr., *J. Food Sci.*, 56(2), 423, 1991.
- Gilman, S., *Electroanalytical Chemistry*, Vol. 2, Bard, A.J., Ed., Marcel Dekker, New York, 1967.
- Gilman, S., *J. Phys. Chem.*, 67, 78, 1963.
- Giner, J., *Electrochim. Acta*, 9, 63, 1964.
- Greenfield, H. and Southgate, D.A.T., *Food Composition Data. Production, Management and Use*, Elsevier Applied Science, London, 1992.
- Hammett, L.P., *J. Am. Chem. Soc.*, 46, 7, 1924.
- Harper, A.E., *Nutr. Today*, 34(6), 216, 1999.
- Hasler, C.M., *Nutr. Rev.*, 54(11, Part II), S6, 1996.
- Henshall, A., High-performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD): a powerful tool for the analysis of dietary fiber and complex carbohydrates, in *Complex Carbohydrates in Foods*, Cho, S.S., Prosky, L., and Dreher, M., Eds., Marcel Dekker, New York, 1999, p. 267.
- Herman, P., *Vegetarian Times*, August 1997.
- Hotchkiss, A.T., Jr. and Hicks, K.B., *Anal. Biochem.*, 184(2), 200, 1990.
- Hotchkiss, A.T., Jr. and Hicks, K.B., *Carbohydr. Res.*, 247, 1, 1993.
- Hotchkiss, A.T., Jr., El-Bahtimy, K., and Fishman, M.L., *Mod. Methods Plant Anal.*, 17, 129, 1996.
- Hughes, S. and Johnson, D.C., *Anal. Chim. Acta*, 132, 11, 1981.
- Hughes, S. and Johnson, D.C., *Anal. Chim. Acta*, 149, 1, 1983.
- Hughes, S. and Johnson, D.C., *J. Agric. Food Chem.*, 30(4), 712, 1982.
- Hughes, S., Meschi, P.L., and Johnson, D.C., *Anal. Chim. Acta*, 132, 11, 1981.
- Ichiki, H., Semma, M., Sekiguchi, Y., Nakamura, M., and Ito, Y., *Nippon Shokuhin Kagaku Gakkaishi*, 2(2), 119, 1996.
- IOM/NAS, *Institute of Medicine/National Academy of Sciences*, Thomas, P.R. and Earl, R., Eds., National Academy Press, Washington, D.C., 1994, p. 109.
- Jago, D., *Prepared Foods*, April 2000.
- Jenkins, D.J.A. et al., *Am. J. Clin. Nutr.*, 71, 401, 2000.

- Johnson, D.C. and LaCourse, W.R., *Anal. Chem.*, 62, 589A, 1990.
- Johnson, D.C. and LaCourse, W.R., *Electroanalysis*, 4, 367, 1992.
- Johnson, D.C. and Polta, T.Z., *Chromatogr. Forum*, 1, 37, 1986.
- Johnson, D.C., *Nature*, 321, 451, 1986.
- Johnson, D.C., Polta, J.A., Polta, T.Z., Neuburger, G.G., Johnson, J., Tang, A.P.C., Yeo, I.H., and Baur, J., *J. Chem. Doc. Faraday Trans. 1*, 82, 108, 1986.
- Katan, M.B., *Lancet*, March 27, 1999.
- Kissinger, P.T., *Laboratory Techniques in Electroanalytical Chemistry*, Kissinger, P.T. and Heineman, W.R., Eds., Marcel Dekker, New York, 1984.
- Koizumi, K., Fukuda, M., Hizukuri, S., *J Chromatogr.*, 585(2), 233, 1991.
- Koizumi, K., Kubota, Y., Tanimoto, T., and Okada, Y., *J. Chromatogr.*, 464(2), 365, 1989.
- Kragten, E.A., Kamerling, J.P., and Vliegthart, J.F.G., *J. Chromatogr.*, 623(1), 49, 1992.
- Kuhn, M.E., *Food Process.*, 59(5), 21, 1998.
- LaCourse, W.R. and Johnson, D.C., *Anal. Chem.*, 65, 50, 1993.
- LaCourse, W.R. and Johnson, D.C., *Carbohydr. Res.*, 215, 159, 1991.
- LaCourse, W.R. and Johnson, D.C., *Advances in Ion Chromatography*, Vol. 2, Jandik, P. and Cassidy, R.M., Eds., Century International Inc., Medfield, MA, 1990.
- LaCourse, W.R. and Owens, G.S., *Anal. Chim. Acta*, 307, 301, 1995.
- LaCourse, W.R., *Analisis*, 21, 181, 1993.
- LaCourse, W.R., Dasenbrock, C.O., and Zook, C.M., *Sem. Food Anal.*, 2, 5, 1997.
- LaCourse, W.R., Jackson, W.A., and Johnson, D.C., *Anal. Chem.*, 61(22), 2466, 1989.
- LaCourse, W.R., Johnson, D.C., Rey, M.A., and Slingsby, R.W., *Anal. Chem.*, 63(2), 134, 1991.
- LaCourse, W.R., Mead, D.A., Jr., and Johnson, D.C., *Anal. Chem.*, 62(2), 220, 1990.
- LaCourse, W.R., *Pulsed Electrochemical Detection in High Performance Liquid Chromatography*, John Wiley & Sons, New York, 1997.
- Larew, L.A. and Johnson, D.C., *Anal. Chem.*, 60(18), 1867, 1988.
- Larew, L.A., Mead, D.A., Jr., and Johnson, D.C., *Anal. Chim. Acta*, 204(1–2), 43, 1988.
- Liu, S. et al., *Am. J. Clin. Nutr.*, 71, 1455, 2000.
- Low, N.H. and Wudrich, G.G., *J. Agric. Food Chem.*, 41(6), 902, 1993.
- Low, N.H., *Am. Lab.*, 28(6), 35M, 1996.
- Lozano, P., Chirat, N., Graille, J., and Pioch, D., *Fresenius J. Anal. Chem.*, 354(3), 319, 1996.
- Lu, Z.X. et al., *Am. J. Clin. Nutr.*, 71, 1123, 2000.
- MacDonald, A. and Duke, P.D., *J. Chromatogr.*, 83, 331, 1973.
- Mannie, E., *Prepared Foods*, June 2000.
- Martens, D.A. and Frankenberger, W.T., Jr., *Chromatographia*, 30(11–12), 651, 1990.
- Martens, D.A. and Frankenberger, W.T., Jr., *Chromatographia*, 30(5–6), 249, 1990.
- Martens, D.A. and Frankenberger, W.T., Jr., *J. Liq. Chromatogr.*, 15(3), 423, 1992.
- Mead, D.A., Jr., Larew, L.A., and Johnson, D.C., *Advances in Ion Chromatography*, Vol. 1, Jandik, P. and Cassidy, R.M., Eds., Century International Inc., Medfield, MA, 1989.
- Messina, M. and Messina, V., *Adv. Exp. Med. Biol.*, 401, 207, 1996.
- Meyer, A., *Food Process.*, 58(8), 32, 1998.
- Meyer, K.A. et al., *Am. J. Clin. Nutr.*, 71, 921, 2000.
- Milner, J.A. and Craig, L., *Am. J. Clin. Nutr.*, 71(Suppl.), 1653S, 2000.
- Milner, J.A., *J. Nutr.*, 129, 1395S, 1999.
- Neuburger, G.G. and Johnson, D.C., *Anal. Chem.*, 60, 2288, 1988.
- Neuburger, G.G. and Johnson, D.C., *Anal. Chim. Acta*, 192, 205, 1990.
- Neuburger, G.G. and Johnson, D.C., *Anal. Chem.*, 59, 203, 1987.
- Ngoviatchai, A. and Johnson, D.C., *Anal. Chim. Acta*, 215, 1, 1988.
- Nutr. Rev.*, 54(11, Part II), S1, 1996.

- Nutr. Today*, 35(2), 66, 2000a.
- Nutr. Today*, 35(3), 72, 2000b.
- Ohr, L.M., *Prepared Foods*, May 1999.
- Ohr, L.M., *Prepared Foods*, October 1998.
- Oku, T., *Nutr. Rev.*, 54(11, Part II), S59, 1996.
- Olechno, J., Carter, S.R., Edwards, W.T., and Gillen, D.G., *Am. Biotechnol. Lab.*, 5, 38, 1987.
- Park, M.K., Park, J.H., Lee, M.Y., Kim, S.J., and Park, I.J., *J. Liq. Chromatogr.*, 17(5), 1171, 1994.
- Parks, E.J. and Hellerstein, M.K., *Am. J. Clin. Nutr.*, 71, 412, 2000.
- Paskach, T.J., Lieker, P.J., and Thielecke, K., *Carbohydr. Res.*, 215, 1, 1991.
- Pastore, P., Lavagnini, I., and Versini, G., *J. Chromatogr.*, 634, 47, 1993.
- Peschet, J.L. and Giacalone, A., *Ind. Aliment. Agric.*, 108(7–8), 583, 1991.
- Pollman, R.M., *J. Assoc. Off. Anal. Chem.*, 72(3), 425, 1989.
- Polta, J.A. and Johnson, D.C., *J. Liq. Chromatogr.*, 6, 1727, 1983.
- Polta, T.Z. and Johnson, D.C., *J. Electroanal. Chem.*, 209, 159, 1986.
- Polta, T.Z., Johnson, D.C., and Luecke, G.R., *J. Electroanal. Chem.*, 209, 171, 1986.
- Prodolliet, J., Bugner, E., and Feinberg, M., *J. Assoc. Off. Anal. Chem.*, 78(3), 768, 1995.
- Quagliani, D., *Better Homes & Gardens*, May 1997.
- Quigley, M.E. and Englyst, H.N., *Analyst*, 117, 1715, 1992.
- Quigley, M.E. and Englyst, H.N., *Analyst*, 119, 1511, 1994.
- Roberfroid, M.B., *Am. J. Clin. Nutr.*, 71(Suppl.), 1682S, 2000.
- Roberfroid, M.B., *Nutr. Rev.*, 54(11, Part II), S38, 1996.
- Rocklin, R.D. and Pohl, C.A., *J. Liq. Chromatogr.*, 6(9), 1577, 1983.
- Sheehy, P.J.A. and Morrissey, P.A., Functional foods: prospects and perspectives, in *Nutritional Aspects of Food Processing and Ingredients*, Henry, C.J.K. and Heppell, N.J., Eds., Aspen Publishers, Gaithersburg, MD, 1998, p. 45.
- Slaughter, L.H. and Livingston, D.P., III, *Carbohydr. Res.*, 253, 287, 1994.
- Smith, S.R. et al., *Am. J. Clin. Nutr.*, 71, 450, 2000.
- Soga, T., Inoue, Y., and Yamaguchi, K., *J. Chromatogr.*, 625, 151, 1992.
- Southgate, D.A.T., *Determination of Food Carbohydrates*, Elsevier, Barking, U.K., 1991.
- Stefansson, M. and Lu, B., *Chromatographia*, 35(1–2), 61, 1993.
- Stuckel, J.G. and Low, N.H., *J. Agric. Food Chem.*, 43(2), 3046, 1995.
- Stulik, W. and Hora, V., *J. Electroanal. Chem.*, 70, 253, 1976.
- Sullivan, J. and Douek, M., *J. Chromatogr.*, 671(1–2), 339, 1994.
- Swallow, K.W. and Low, N.H., *J. Agric. Food Chem.*, 38(9), 1828, 1990.
- Swallow, K.W. and Low, N.H., *J. Assoc. Off. Anal. Chem. Int.*, 77(3), 695, 1994.
- Swallow, K.W., Low, N.H., and Petrus, D.R., *J. Assoc. Off. Anal. Chem.*, 74(2), 341, 1991.
- Tengyl, J., *Electrochemical Detectors*, Ryan, T.H., Ed., Plenum Press, New York, 1984.
- Thompson, F.E. et al., *Am. J. Clin. Nutr.*, 71, 1503, 2000.
- Trowell, H., Dietary fibre and coronary heart disease, *Rev. Eur. Etud. Clin. Biol.*, 17(4), 345, 1972.
- Turner, L., *Vegetarian Times*, February 1999.
- U.S. Senate Select Committee on Nutrition and Human Needs, *Dietary Goals for the United States*, 2nd ed., U.S. Government Printing Office, Washington, D.C., 1977.
- Van Nifterik, L., Xu, J., Laurent, J.L., Mathieu, J., and Rakoto, C., *J. Chromatogr.*, 640(1–2), 335, 1993.
- Van Riel, J. and Olieman, C., *Carbohydr. Res.*, 215, 39, 1991.
- Van Rooijjan, H.W. and Poppe, H., *Anal. Chim. Acta*, 130, 91, 1991.
- Vandeberg, P.G., Kowagoe, J.L., and Johnson, D.C., *Anal. Chim. Acta*, 260(1), 1, 1992.
- Wagner, H.P. and McGarrity, M.J., *J. Am. Soc. Brew. Chem.*, 50, 1, 1992.

- Wagner, H.P., *J. Am. Soc. Brew. Chem.*, 53, 82, 1995.
- Walqvist, M.L., Functional foods in the control of obesity, in *Functional Foods: Designer Foods, Pharmafoods, Nutraceuticals*, Goldberg, I., Ed., Chapman & Hall, London, 1994, p. 71.
- Wang, W.T. and Zopf, D., *Carbohydr. Res.*, 189, 1, 1989.
- Weisz, J. et al., Poster #AAFC 052, *Food Science at Work: Technical Communications from The Guelph Food Research Community*, September 16, Guelph, Ontario, Canada, 1999.
- Welch, L.E., LaCourse, W.R., Mead, D.A., Jr., and Johnson, D.C., *Anal. Chem.*, 61, 555, 1989.
- Welch, L.E., LaCourse, W.R., Mead, D.A., Jr., and Johnson, D.C., *Talanta*, 37, 377, 1990.
- White, D.R., Jr. and Widmer, W.W., *J. Agric. Food Chem.*, 38(10), 1918, 1990.
- White, D.R., Jr. and Cancalon, P.F., *J. AOAC Int.*, 75(3), 584, 1992.
- Wilson, R., Cataldo, A., and Andersen, C.P., *Can. J. For. Res.*, 25(12), 2022, 1995.
- Wong, K.S. and Jane, J., *J. Liq. Chromatogr.*, 18, 63, 1995.
- Zeisel, S.H., *Science*, 285, 1853, 1999.
- Zook, C.M., Patel, P.M., LaCourse, W.R., and Ralapati, S., *J. Agric. Food Chem.*, 44(7), 1773, 1996.
- Zook, C.M. and LaCourse, W.R., *Curr. Sep.*, 14(2), 48, 1995.

9 Analysis of Flavonoids

Gowsala Sivam

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9.1 INTRODUCTION

Three of the most important natural pigments are carotenoids, tetrapyrrole derivatives, and flavonoids. The flavonoids are plant polyphenols found frequently in fruits, vegetables, and grains. They are responsible for the color of flowers, fruits, and sometimes leaves. When not directly visible, they often act as copigments. For example, colorless copigments protect anthocyanins from damage caused by ultra-violet (UV) radiation. They also protect plant tissues from the effects of UV radiation. It is variations of the structure of anthocyanins that generate the wonderful diversity of colors in flowers and fruits. The subtleties of tones and hues in cranberries, blueberries, blackberries, strawberries, and boysenberries are thought to result from a number of factors:



FIGURE 9.1 Numbering of flavonoid skeletons.

- Differences in electron charge
- Number of methoxyl and hydroxyl groups
- Number and type of sugars attached to the aglycone
- Presence of metal ions
- Presence of other pigments

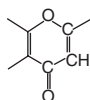
Structurally, flavonoids are derivatives of 1,3-diphenyl propane as shown in Figure 9.1. One of the benzene rings, ring A, originates from acetate and is formed by a ring closure of a polyketide. The other ring, ring B, is derived from the shikimic acid pathway (Samuelsson, 1999). One hydroxyl group in ring A is practically always situated in *ortho*-position to the side chain. Ring closure involving the 3-carbon chain and this hydroxyl group can form the third 6-membered ring characteristic of most of the flavonoids. Flavonoids occur both in free state (aglycones) and as glycosides. They differ in their substituents, mostly hydroxyl or methoxyl groups, and in the nature and position of the sugar residues. Over 4000 flavonoids have been reported (Strack and Wray, 1994), and they form the largest group of naturally occurring polyphenols. The most common classes are the flavones, flavonols, anthocyanidins, flavanones, catechins, and isoflavonoids (Figure 9.2). Together these classes represent more than 80% of the known flavonoids. Harborne and colleagues have edited several volumes on the science of the flavonoids, the most recent of which is a 15-chapter book (Harborne and Grayer, 1994).

9.2 COMMON CLASSES OF FLAVONOIDS

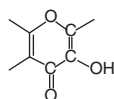
Anthocyanins are glycosides and acylglycosides of anthocyanidins. They are usually C3 monosides, biosides, and triosides, although there are also 3,5- and 3,7-diglycosides (Strack and Wray, 1994). There are six anthocyanidins commonly found in fruits (Figure 9.3) (Goiffon, Brun, and Bourrier, 1991). The most common is cyanidin. Blueberries contain all but pelargonidin (Robards and Antolovich, 1997). Most of the red color of strawberries is due to pelargonidin 3-glucoside, but strawberries also contain cyanidin 3-glucoside (Bakker, Bridle, and Koopman, 1992). Pomegranate juices with delphinidin as the main anthocyanidin are violet and those with pelargonidin are scarlet (Gil et al., 1995a).

Catechins are found mainly in brewed tea (Bronner and Beecher, 1998) and in red wine (Goldberg et al., 1998). The concentration of catechins is higher in green tea than in black and oolong teas, which are partially oxidized (Khokhar et al., 1997). Figure 9.4 shows the common catechins.

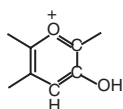
Structure of central C-3 unit



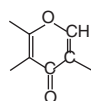
Flavone



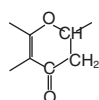
Flavonol



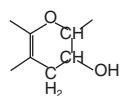
Anthocyanidin



Isoflavone

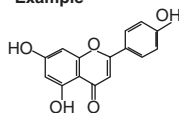


Flavanone

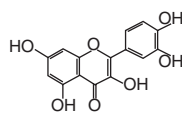


Catechin

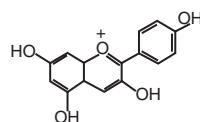
Example



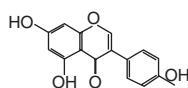
Apigenin



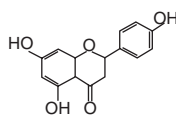
Quercetin



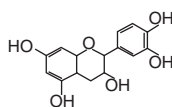
Pelargonidin



Genistein



Naringenin



Catechin

FIGURE 9.2 Flavonoid categories.

Flavanones (Figure 9.5) are predominant in citrus, where they exist as mono- and diglycosides. Naringin and neohesperidin have been found in grapefruit juices and are important for quality control and bitterness. Hesperidin and neohesperidin

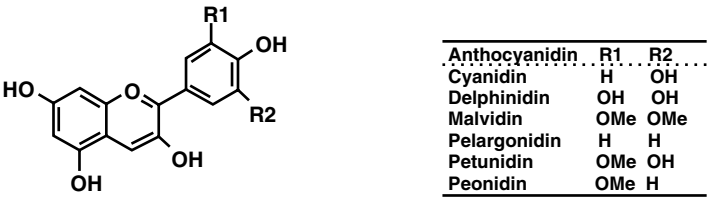


FIGURE 9.3 Anthocyanidin skeleton.

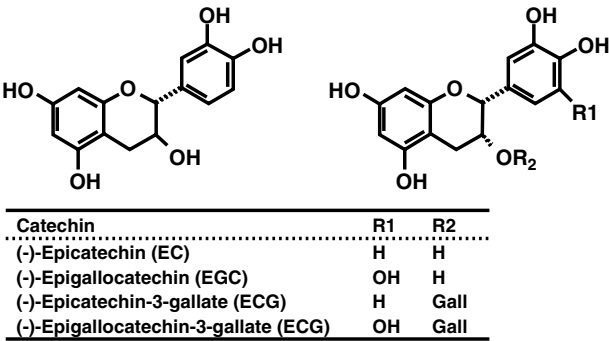


FIGURE 9.4 Catechin skeleton.

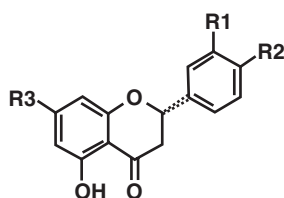
have been reported in common sweet oranges. High concentrations of eriocitrin and neoeriocitrin have been found in lemon juices and sour oranges, respectively (Mouly, Gaydou, and Estienne, 1993; Mouly et al., 1994). Honey and propolis also contain flavanone glycosides.

Flavones and flavonols (Figure 9.6) are usually found in plants as *O*-glycosides. The flavonols have a hydroxyl at C3, whereas the flavones have a hydrogen (Figure 9.3). Glycosides of the flavonol quercetin predominate in vegetables, and glycosides of flavonol kaempferol and of the flavones apigenin and luteolin also exist in vegetables. In fruits, glycosides of quercetin are usually the only flavonols found, with glycosides of myricetin and kaempferol existing in trace amounts (Hertog, Hollman, and Venema, 1992).

Isoflavones (Figure 9.7) are predominantly found in soy and its products (Mazur et al., 1998). At least 15 isoflavones have been reported from food, usually as glycosides, although aglycones are found in fermented soy products. Low levels of isoflavone are found in other legumes (Bingham et al., 1998).

9.2.1 FLAVONOIDS IN PLANT PRODUCTS

Table 9.1 provides the total polyphenolic content of plant foods and beverages. Table 9.2 provides the flavonoid contents in selected plant foods.



Flavanone	R1	R2	R3
Didymin	H	OMe	ORut ^a
Eriocitrin	OH	OH	ORut
Eriodictyol	OH	OH	OH
Hesperetin	OH	OMe	OH
Hesperidin	OH	OMe	ORut
Isocakuranetin	H	OMe	OH
Naringenin	H	OH	OH
Naringin	H	OH	ONeo ^b
Narinnutin	H	OH	ORut
Neeriocitrin	OH	OH	ONeo
Neobesperidin	OH	OMe	ONeo
Pinocembrin	H	H	OH
Poncirin	H	OMe	ONeo
Prunin	H	OH	OGlu ^c

^arutinoside; ^bneohesperidose; ^cglucose

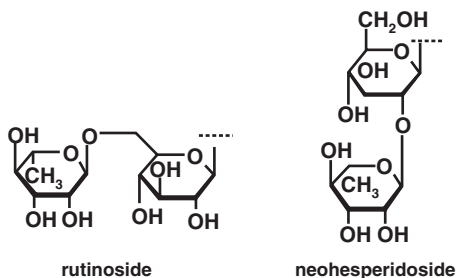


FIGURE 9.5 Flavanone skeleton.

9.3 BIOLOGICAL ACTIVITY OF FLAVONOIDS

The first suggestion of biological activity of flavonoids was presented by Szent-Gyorgyi in 1938, who reported that citrus peel flavonoids were effective in preventing capillary bleeding and fragility associated with scurvy. Since then a great number of pharmacological effects have been ascribed to flavonoids. The following activities have been described: anti-inflammatory, antihepatotoxic, antitumor, antimicrobial, antiviral, enzyme inhibiting, antioxidant, and central vascular system effects. Formica and Regelson (1995) reviewed the biological activity of flavonoids, and Rice-Evans, Miller, and Paganga (1996) reviewed the structure–antioxidant activity relationships. Most of the pharmacological investigations of flavonoids have been performed *in vitro*, and in most cases it is not known if the concentrations giving the observed effects are therapeutically realistic. As very few pharmacokinetic studies

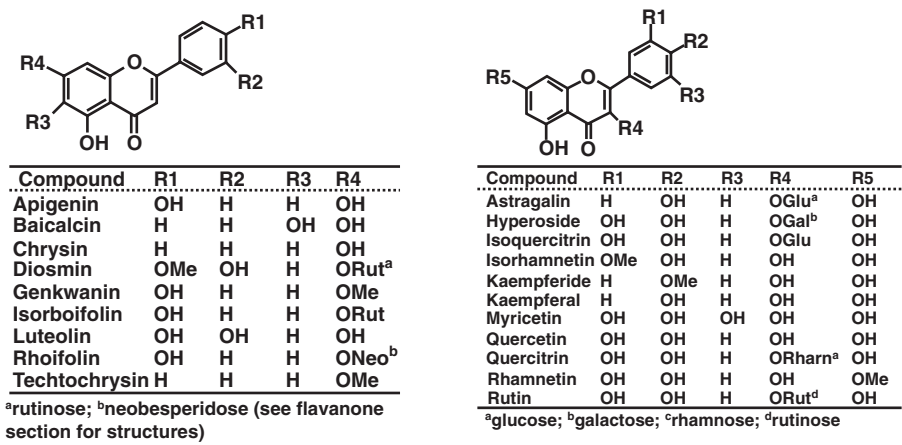


FIGURE 9.6 Flavone and flavonol skeletons.

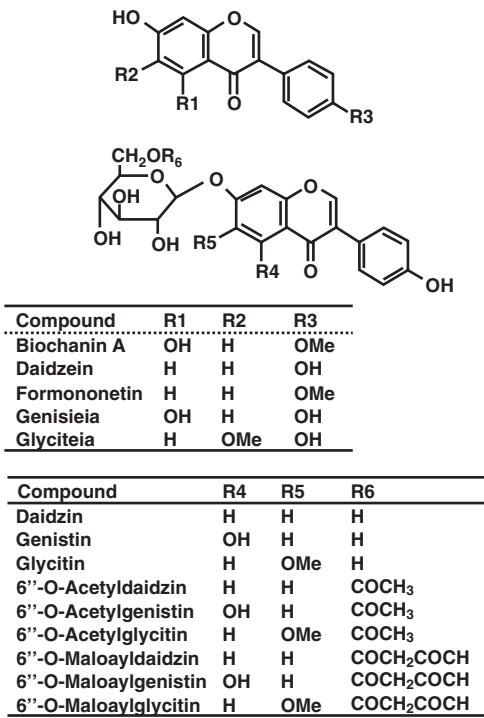


FIGURE 9.7 Isoflavone skeleton.

have been performed, one does not know whether the flavonoids arrived at the alleged site of action in the native state and in sufficient concentrations. Health implications require detailed knowledge of the flavonoid content of the food supply

TABLE 9.1
Total Polyphenolic Content of Plant Foods and Beverages

Food ^a	Total Polyphenols	Food/Beverage	Total Polyphenol
Cereals (mg/100 g dm)		Fruits (mg/100 fm)	
Barley	1200–1,500	Blackcurrant	140–1,200
Corn	30.9	Blueberry	135–280
Millet	590–1,060	Cherry	60–90
Oats	8.7	Apple	27–298
Rice	8.6	Cranberry	77–247
Sorghum	170–10,260	Gooseberry	22–75
Wheat	22–40	Grape	50–490
Legumes		Grape fruit	50
Black gram	540–1,200	Orange	50–100
Chickpeas	78–230	Pear	2–25
Cowpeas	175–590	Plum	4–225
Common beans	34–280	Raspberry	37–429
Green gram	440–800	Red currant	17–20
Pigeon peas	380–1,710	Strawberry	38–218
Nuts (% dm)		Tomato	85–130
Betel nuts	26–33	Fruit juices (mg/l)	
Cashew nuts	33.7	Apple juice	2–16
Peanuts	0.04	Orange juice	370–7,100
Pecan nuts	8–14		
Vegetables (mg/100 g fm)		Beverages	
Brussels sprouts	6–15	Tea (mg/200 ml)	150–210
Cabbage	25	Coffee (mg/150 ml)	200–550
Leek	20–40	White wine (mg/l)	200–300
Onion	100–2,025	Red wine (mg/l)	1,000–4,000
Parsley	55–180	Beer (mg/l)	60–100
Celery	94		

^a dm, dry matter; fm, fresh matter.

as well as concentrations achievable in the plasma, hence, this chapter on the analytical methods on the measurement of flavonoids.

9.4 ANALYTICAL METHODS ON THE MEASUREMENT OF FLAVONOIDS

9.4.1 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) OF FLAVONOIDS

Computer-controlled HPLC has become the analytical method of choice for flavonoids. HPLC was first used for the determination of flavonoids in 1976 by Fisher and Wheaton (Hasegawa, Berhow, and Fong, 1996). Since then many systems have been developed for the detection and quantification of flavonoids across one, two, or three subclasses. There have been several reviews of the HPLC methods used so far. Daigle and Conkerton (1983, 1988) reviewed the HPLC analysis of flavonoids in 1983 and subsequently in 1988. Robards and Antolovich (1997) reviewed chromatographic

TABLE 9.2
Flavonoids in Selected Plant Foods

Flavonoid	Plant Products
Isoflavones	
Daidzin, genistin, daidzein, genistein.	Soya bean
Flavonols	
Quercetin mono- and diglucoside	Onion
Quercetin, Kaempferol	Tea
Anthocyanins	
Cyanidin glucosides	Red currant
Delphinidin, malvidin, cyanidin, petunidin, peonidin	Blueberry
Pelargonidin	Strawberry
Flavanones	
Naringin, neohesperidin	Grape fruit
Hesperidin, neohesperidin	Sweet orange
Eriocitrin, neoeriocitrin	Lemon, sour orange
Catechins	
Catechins/epicatechins	Grape skin, tea

methods of detection of flavonoids and concentrated on the HPLC methods published from 1989 to early 1997. The methodology is divided into anthocyanins, catechins, flavanones, flavones and flavonols together, and isoflavones. This chapter also has a section devoted to HPLC methods utilized for flavonoids across two or more subclasses. The most recent review is by Merken and Beecher (2000a). A summary of the various HPLC and sample preparation methods that have been employed to quantify individual flavonoids within a subclass or across several subclasses is tabulated in this review (Merken and Beecher, 2000a). The authors have tabulated the following: food, sample preparation, guard column, stationary phase, mobile phase, and references; this is an excellent review for recent HPLC methods used for flavonoids. Methods reviewed in this publication and the references cited therein are summarized here.

9.4.1.1 Sample Preparation

Syrups and wines required no sample preparation. Teas were boiled, and sometimes solid-phase extraction (SPE) and/or liquid–liquid extractions (LLE) were then used. Countercurrent chromatography and solid-phase columns such as Sephadex LH-20 and Sep-Pak were also used (Amarowicz and Shahidi, 1996; Liang et al., 1990). Extractions to remove lipids, carotenoids, and chlorophyll were used with French apple cider (Guyot et al., 1998). For citrus extracts, extractions were done to remove carotenoids and methoxy flavones (Marini and Balestrieri, 1995). For honey, usually SSE was used (Bogdanov, 1989).

Berries were homogenized (Gao and Mazza, 1994, 1995). Pomegranate seeds were crushed (Gil et al., 1995a,b). Black beans were pulverized by mortar and pestle (Takeoka et al., 1997). LLE or SPE was utilized for onions and potatoes (Donner,

Gao, and Mazza, 1997; Rodriguez-Saona, Gousti, and Wrolstad, 1998). Soy extract required hydrolysis with acid (Hutabarat, Mulholland, and Greenfield, 1998).

9.4.1.2 Hydrolysis

Glycosides of flavonoids frequently required hydrolysis to remove the sugar moiety. Acidic, basic, or enzymatic hydrolysis was used. Since anthocyanin samples are difficult to obtain, anthocyanins were often hydrolyzed to anthocyanidins and hydrolysis was typically carried out in refluxing HCl solutions such as 50% MeOH–2 *N* HCl (v/v) (Gao and Mazza, 1994). Similar conditions have been used for glycosides of flavones and flavonols. Alkaline hydrolysis was used for acylated anthocyanins (Hong and Wrolstad, 1990), and a technical enzyme (EL-1-77; Rohm, Darmstadt, Germany) was used in the hydrolysis of flavonol glycosides (Finger, Engelhardt, and Wray, 1991a).

9.4.1.3 Chromatographic Conditions

Reversed-phase columns (RP) are the norm, ranging from 100 to 300 mm in length with a 4.6-mm ID. Stereochemical separation is rarely an issue, a β -cyclodextrin-bonded stationary phase (Cyclobond 1) was used to separate the 2*R* and 2*S* diastereomers of flavanone glycosides (Krause and Galensa, 1990).

Elution systems are usually binary, with an aqueous acidified solvent (solvent A) such as aqueous acetic acid, perchloric acid, or formic acid and an organic solvent such as methanol or acetonitrile, possibly acidified (solvent B). Trifluoroacetic acid in both solvents enhances the resolution and eliminates peak tailing of catechins (Dalluge et al., 1998). Elution systems are less frequently isocratic or tertiary, and even quarternary systems have been reported (de Pascual-Teresa et al., 1998). Duration of the HPLC runs are generally 1 h maximum, with equilibration between runs. Flow rates are usually 1.0 to 1.5 ml/min. Columns are usually maintained close to ambient temperature and the injection volume generally ranges from 1 to 100 μ l.

9.4.1.4 Detection

UV-visible (UV-vis) with diode array detection (DAD) is the standard method used for detection of flavonoids. Since flavonoids are polyphenols, two UV absorption bands are characteristic of this class of compounds. Band II, with a maximum in the 240- to 285-nm range, is believed to arise from the A ring. Band I, with a maximum in the 300- to 550-nm range, presumably arises from the B ring. Mabry et al. (1970) have published the UV spectra of 175 flavonoids and their molecular extinction coefficients. Figure 9.8 shows UV-vis spectra representative of the major classes of flavonoid aglycones. In general, UV spectra of flavanones, isoflavones, and catechins have an intense band II peak and a weak band I peak because of little or no conjugation between A and B rings. A fluorescence detector (280 nm for excitation, 310 nm for emission) connected in series with a UV detector (270 nm) has also been used (Arts and Hollman, 1998). Derivatization with *p*-dimethylaminocinnamaldehyde resulting in colored adducts with catechins was also reported, allowing detection at 640 nm (de Pascual-Teresa et al., 1998). Higher sensitivity has

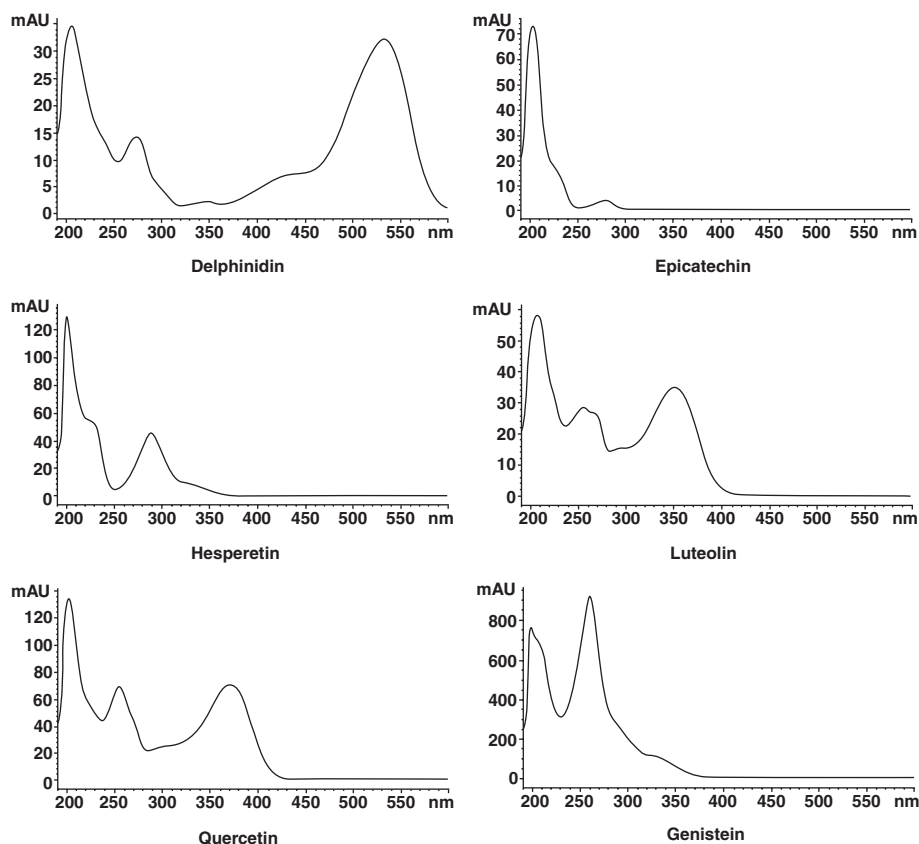


FIGURE 9.8 UV-vis spectra of the anthocyanidin delphinidin, the catechin epicatechin, the flavanone hesperetin, the flavone luteolin, the flavonol quercetin, and the isoflavone genistein.

been achieved for detection of green tea extracts by using chemiluminescence (Ogawa et al., 1999).

Several hundred papers on the HPLC of flavonoids have been published in the past two decades, yet these methods can detect flavonoids across one, two, or perhaps three subclasses in one run. Foods may contain several subclasses, and a mixed diet contains all subclasses. A method is necessary to simultaneously measure all prominent flavonoids in food and drink. This separation has been achieved recently by Merken and Beecher (2000b). They have developed an HPLC system for the separation and quantification of 17 flavonoids, as their aglycones, which represent all 5 subclasses and are expected to be prominent in commonly consumed foods.

The utility of HPLC is best illustrated by an example such as that shown in Figure 9.9. In this experiment a mixture of flower extracts has been used to present a broad spectrum of compound type. Detection in the range of 340 to 360 nm is suitable for flavones and flavonols. In the crude mixture, the series of peaks can be grouped based on the on-line spectra (Figure 9.9a). HPLC analysis of the mixture after alkaline hydrolysis (removes any acyl groups) (Figure 9.9b) and acid hydrolysis

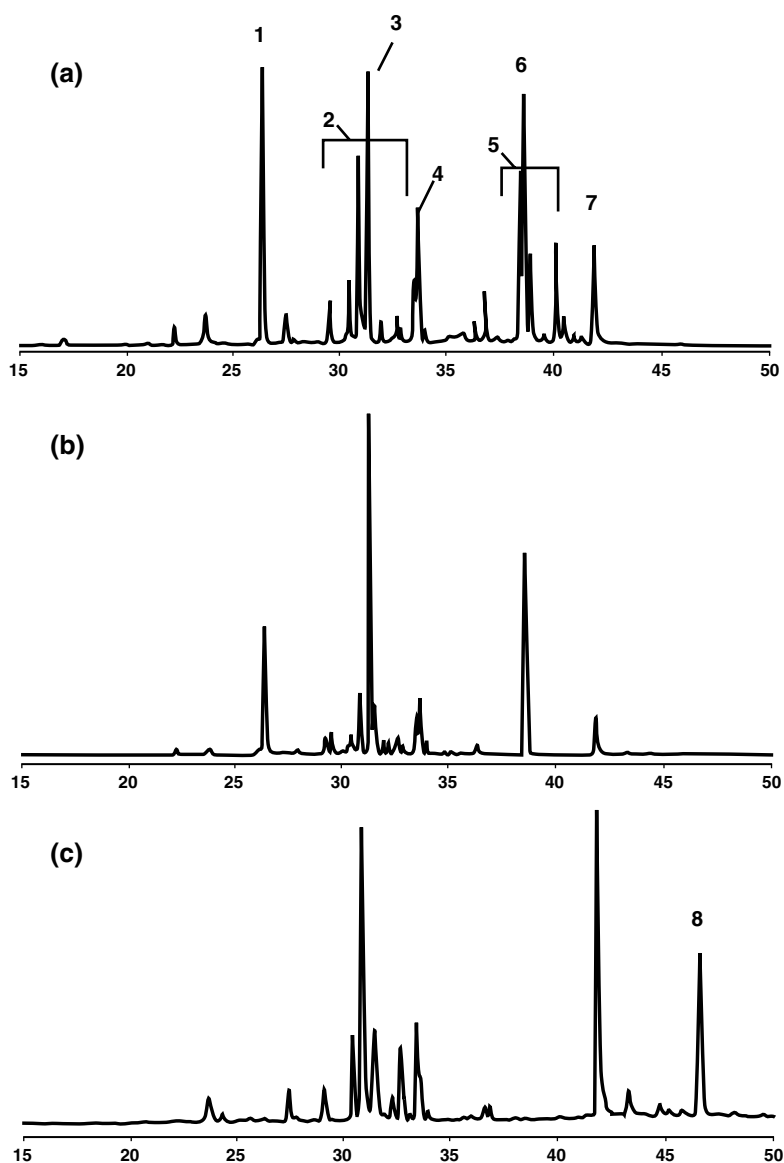


FIGURE 9.9 HPLC chromatograms [absorbance at 352 nm vs. time (min)]. (a) A flavonoid mixture showing different groups of compounds: a kaempferol triglycoside (1), a set of apigenin glycosides (2), a kaempferol diglycoside (3), a luteolin glycoside (4), a set of acylated kaempferol glycosides (5), a chalcone (6), and luteolin (7). (b) The alkaline hydrolysis product of the same mixture shows a large relative increase in peak 3 and loss of the acylated kaempferol glycoside peaks. (c) The acid-hydrolyzed mixture showing luteolin and kaempferol (8). Peaks due to apigenin glycosides are still present, showing these are apigenin C-glycosides.

(cleaves *O*-glycosides) (Figure 9.9c) helps in the identification of the peaks. Quantification of flavonoids is another advantage of HPLC in combination with UV detection — a rough estimate of the flavonoid level can be arrived at by comparing integration data for the chromatogram with that from the injection of a known amount of a readily available standard.

9.4.2 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION METHODS

As mentioned earlier, flavonoids present in plant foods belong to six different classes, and within one class, they can vary from one another subtly in the degree of unsaturation, the pattern of hydroxylation or methylation, and the type of glycosylation. Considering such complexity, even the best HPLC methods cannot resolve and separate all flavonoid compounds from complex mixtures. For the HPLC analysis described so far, photodiode array detectors have been the method of choice. These detectors facilitate, and with the help of on-line UV spectra they can help identify a compound, if it is cleanly resolved. Often the chromatography of complex mixtures is plagued with coelution of absorbing materials other than flavonoids. Since flavonoids are known to have anti-oxidant properties, and the essence of redox chemistry involves electron transfer, electrochemical detection would be a suitable method of detection when combined with UV-vis detection and may significantly increase the the potential of HPLC analysis. Milbury (2001) has written a concise review on this topic.

Analyte detection is accomplished using 16 channels of a coulometric electrode array system (CEAS) (ESA, Inc., Chelmsford, MA) incremented from -100 to 990 mV vs. palladium. In the coulometric electrodes utilized in the CEAS, Coularray instruments are constructed from porous graphite, and the instrument flow stream is directed through the electrode rather than over it. This method permits efficiencies of nearly 100% analyte oxidation, and lower detection limits are thus possible. Data generated from an electrochemical array can be thought of in much the same way as those obtained from a photodiode array. Analytes are resolved by chromatographic retention time while the current is monitored from each detector in the series, and each detector can be set to distinct redox potentials. A compound entering the array is oxidized upon reaching the first electrode in the array that is set to a redox potential capable of oxidizing it. When an array is configured with increasing potentials, a three-dimensional retention time/redox potential/current response display can be generated (Figure 9.10a). If resolved cleanly, each analyte will exhibit a characteristic retention time and a response across the array as shown for catechin in Figure 9.10b. When a pure standard analyte is available, setting the array at increments of less than 10 mV permits very accurate determination of the redox potential vs. palladium at the pH of the mobile phase.

9.4.3 MASS SPECTROMETRY

Mass spectrometry (MS) is one of the physicochemical methods applied to the structural determination of organic compounds. The high sensitivity and possibility

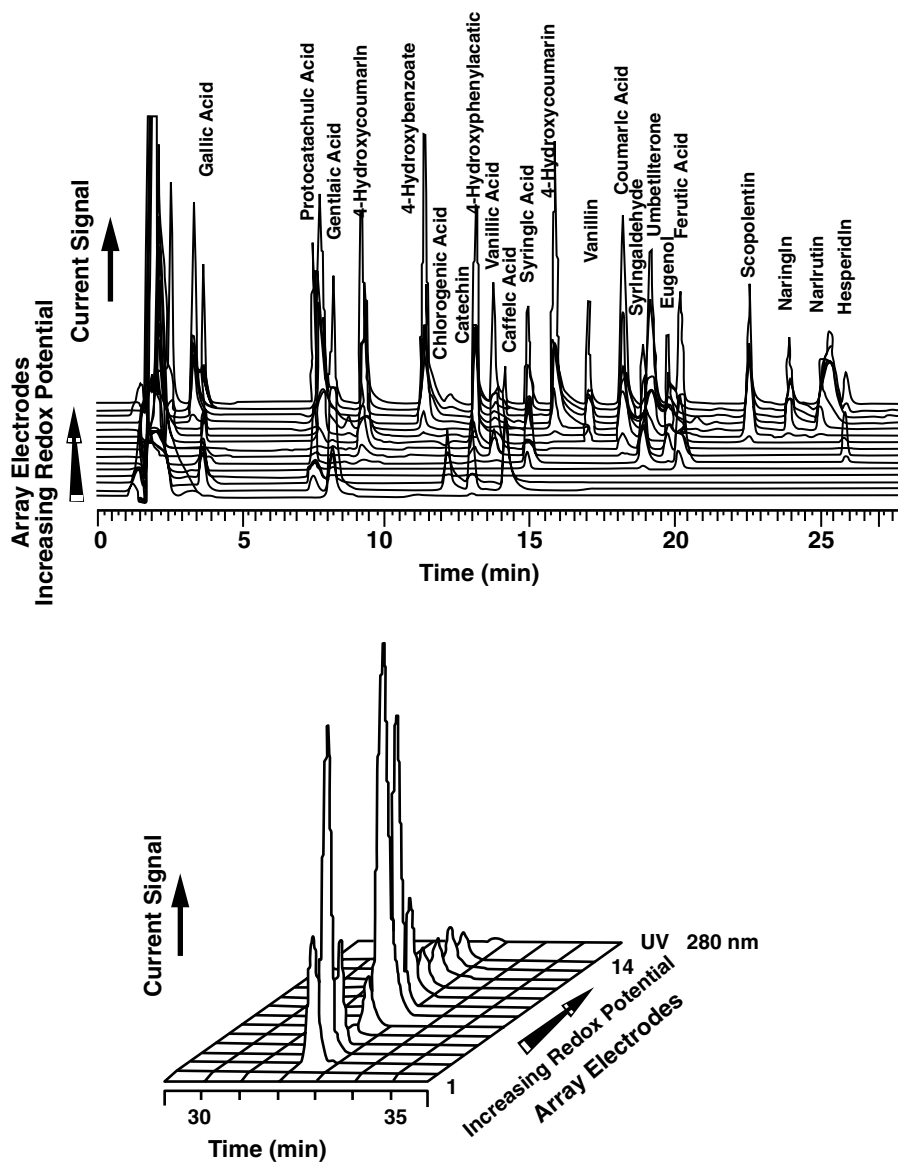


FIGURE 9.10 (a) A 15-channel chromatogram from the CEAS instrument. C_{18} column used with mobile phase A, 0.1 M sodium phosphate and 10 mg/l sodium dodecyl sulfate (SDS) at pH 3.35; mobile phase B, 0.1 M sodium phosphate and 50 mg/l SDS at pH 3.35 in methanol-acetonitrile (30:10:60, v/v/v). The array increases from 0 to 840 mV and is displayed from the lower to the upper trace. The vertical response represents the current signal in nanoamperes. (b) A three-dimensional representation of retention time, array channel potentials, and channel current response for catechin.

of hyphenation with chromatographic techniques sets MS among the most appropriate physicochemical methods for the study of natural products from biological material. The characteristic features of MS are the use of different physical principles, both for sample ionization and for separation of the ions. An excellent review has been written by Stobiecki (2000) on the application of MS for identification and structural studies of flavonoid glycosides. The application of MS to the analysis of flavonoid glycosides has increased with the so-called “soft” ionization techniques. Compounds of this class are polar, nonvolatile, and thermally labile. Electron impact (EI), with electron energies ranging from 10 to 100 eV, and chemical ionization (CI) were not suitable for MS analyses of underivatized flavonoid glycosides. Both methods require the analyte to be in the gas phase for ionization, and derivatization of the hydroxyl groups was necessary. The introduction of thermospray (TSP) and electrospray interfaces (ESI) allowed direct coupling of MS with liquid chromatography (LC), and this permitted studying flavonoid-containing plant products. Both TSP and ESI are soft ionization techniques and allow the analysis of flavonoids in their native form without derivatization. Arpino (1989, 1990) has described the LC-MS techniques introduced between 1980 and 1989 in an excellent review. In addition, a number of papers that deal with the advances in instrumentation and applications of LC-MS systems for the analysis of mixtures of secondary metabolites from plants have been published (see Niessen, 1998).

Flavonoid aglycones are structurally a rather diverse group of natural products. The most important variations in their structure arise from the level of oxygenation (hydroxyl or methoxyl groups) and the point of attachment of ring B (flavonoids and isoflavonoids). The sugar component may consist of hexoses, deoxyhexoses, or pentoses and, in some cases, glucuronic acids with the added possibility of *O*- or *C*-glycosidation. With respect to flavonoid glycosides, the following information can be obtained from mass spectra: (1) molecular mass, (2) structure of aglycone (pattern of hydroxylation, point of attachment of ring B on ring C), (3) information about acylation of sugar hydroxyl groups and methylation of aglycone hydroxyl group(s), and (4) number of sugars and in some cases placement of glycosidic bonds. MS does not provide information about stereochemistry of the glycosidic linkage or distinguish between diastereomeric sugar units. Figures 9.11a and 9.11b give the MS of quercetin 3-*O*-galactoside 7-*O*-glucoside, unmodified and permethylated, respectively.

HPLC has been coupled to a mass spectrometer interface, as was done in the analysis of fruits, vegetables, and beverages (Justesen, Knuthsen, and Leth, 1998). Fast atom bombardment mass spectrometry (FABMS) of pelargonidin 3-glycoside in strawberry puree collected after HPLC confirmed its identity (Bakker, Bridle, and Koopman, 1992). HPLC-quadrupole MS was used for detecting flavones in olive oil (Rovellini, Cortesi, and Fedeli, 1997). Lin, Ng, and Yang (1993) found the expected (–)epicatechin, (–)epicatechin 3-*O*-gallate, (–)epigallocatechin, and (–)epigallocatechin 3-*O*-gallate in tea using tandem MS following HPLC or LC-MS-MS. Thus, HPLC procedures with UV detection and MS were developed to further characterize the flavonoids. More recently, using HPLC coupled to a DAD, and in parallel to ESI-MS, a methodology has been developed to observe flavonols directly

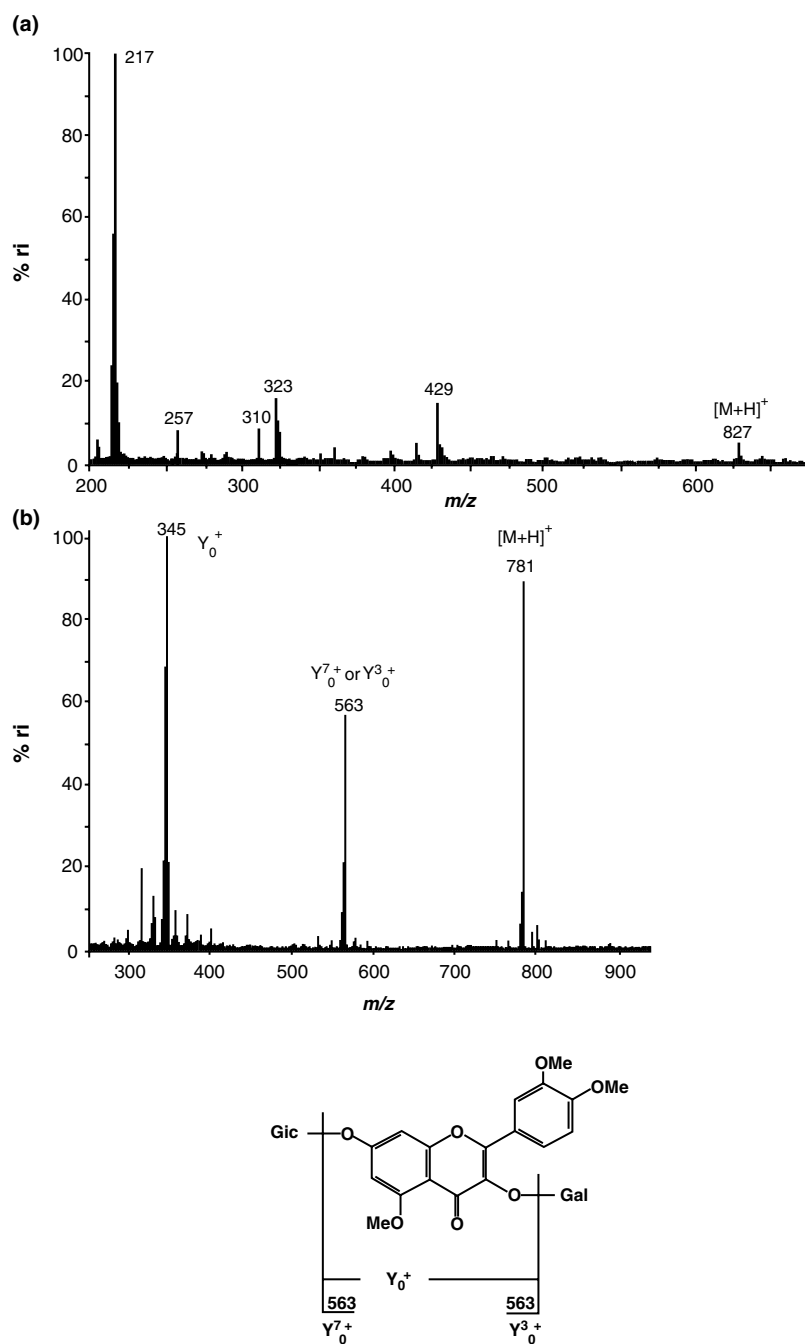


FIGURE 9.11 MS of quercetin 3-*O*-galactoside 7-*O*-glucoside, unmodified (a) and permethylated (b).

in tomato puree extracts (Mauri et al., 1999). By this method, it has been possible to detect intact flavonol glycosides in tomato extracts and to characterize a flavonol trisaccharide. For the first time using LC-ESI-MS, it has been possible to detect intact flavonol glycosides in the plasma of healthy volunteers and to provide further evidence on the absorption of flavonoid glycosides after consumption of common vegetables such as tomatoes.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is a new technique that is having a great impact on food analysis. Recently, it has been demonstrated that this technique is useful to identify flavonol glycosides in food samples, tea, and yellow onion bulbs (Wang and Sporns, 2000). MALDI-TOF-MS was first introduced in 1987 and was originally developed for large biomolecules. This method has advantages over other methodologies, including speed of analysis, high sensitivity, wide applicability combined with a good tolerance toward contaminants, and the ability to analyze complex mixtures. Potential application of MALDI-TOF-MS in food products allows for analysis of most flavonoid molecules. Presently, its applications in this area are limited; it has been reported for both qualitative and quantitative analysis of anthocyanins and flavonol glycosides (Wang and Sporns, 1999, 2000).

With appropriate derivatization, flavonoids can be analyzed by a combination of gas chromatography-mass spectrometry (GC-MS). It is now becoming common knowledge in the GC community that high-resolution GC (HRGC) can be used to analyze many compounds traditionally considered intractable as a result of being polar or "thermolabile." This knowledge, however, has not been completely disseminated to the different domains of chemical analyses, and, hence, HPLC has been the preferred method of analysis. Yet in many cases, HRGC has distinct advantages when compared to HPLC; it offers high resolution, faster separation, ease of coupling with a wide variety of detectors, no use of solvents, etc. Today, HRGC coupled with MS (HRGC-MS) is an important and consolidated method for the systematic analysis of natural products including flavonoids (Carbonell et al., 2000).

9.4.4 THIN-LAYER CHROMATOGRAPHY

Flavonoids are a very important class of natural products, and therefore, many techniques have been used to identify and quantify these compounds. Early investigations were carried out using paper chromatography and thin-layer chromatography (TLC). Historically, paper chromatography has been the preferred method for flavonoid analysis, and relative mobility data are available for a large variety of compounds. However, currently, very few laboratories are equipped to perform paper chromatography, but TLC is a popular alternative because of its speed and versatility resulting from the availability of a wide range of stationary adsorbent phases (alumina, silica, cellulose, polyamide, reverse-phase silica, etc.). This analytical tool is especially useful for quick analysis of material containing flavonoids, particularly flavonoid glycosides. Bloor (2001) recently summarized the TLC analysis of this class of compounds. Literally, there is an adsorbent and eluent combination to suit every conceivable type of flavonoid. The range of adsorbent/eluent combination enables the chromatography to be carried out in several totally different systems,

thereby leading to more reliable analytical data and comparisons of data. Typically, plastic-backed sheets of the adsorbent phase are cut to a suitable size, samples are applied as 1- to 2-mm spots and allowed to dry, and the chromatogram is developed in a glass tank equilibrated with the mobile phase. Two solvent systems are routinely used as the mobile phase for preliminary analyses: 15% acetic acid (acetic acid–water, 15:85) and TBA (*t*-butanol–acetic acid–water, 3:1:1). The relative mobilities in TBA and 15% acetic acid should give a rough guide as to flavonoid type. Developed chromatograms are dried and viewed under UV light (366 nm) or sprayed with suitable reagents. If necessary, a fingerprint type of two-dimensional chromatogram can be performed as well. Suitable solvent systems and spray reagents are tabulated in the review article (Bloor, 2001).

9.4.5 CAPILLARY ZONE ELECTROPHORESIS

More modern separation systems have been used to a limited extent to analyze flavonoids. Capillary zone electrophoresis (CZE) is a relatively new technique with considerable potential for analyzing charged compounds and has been used to analyze several flavonoids in plant extracts. It is a new refinement of traditional electrophoresis in which separating power has increased to an extent (up to 120,000 theoretical plates) that it now exceeds HPLC by up to 65 times (Markham and Bloor, 1998; Stenner et al., 1990). Sensitivity is also 10 to 12 times higher than in HPLC, and the sample size injected is about 1 ng. CZE is a hybrid of chromatography and electrophoresis. HPLC, however, remains the method of choice for flavonoid analysis. However, CZE offers a method for separating flavonoids that utilizes quite different molecular properties. As such, CZE will sometimes produce separations where HPLC will not.

Issaq (1999) reviewed the use of capillary electrophoresis of natural products. Aussenac, Lacombe, and Dayde (1998) optimized the conditions for separation of isoflavones in soy bean extracts using CZE. The separation mechanism in CZE is based on differences in the electrophoretic mobilities of molecules, and thus, flavonoids not carrying a charge must be ionized by using a suitable buffer. Borate buffers with a pH of 8 to 11 and a concentration of 25 to 200 mM are commonly used, although this buffer can lead to complexation in the case of flavonoids with vicinal or *ortho*-dihydroxyl groups. In such cases, tris-HCl and phosphate groups have been substituted. Flavonoid compounds being phenols are weak acids. The migration time depends on the charge-to-size ratio; total migration time for highly charged small flavonoid molecules is longer than that for molecules of smaller charge and larger size, because the patterns of phenol and sugar substitution differ (McGhie, 1993). As is typical in CZE, the pH of the buffer has a marked effect on total migration time and on the separation of all the isoflavones in the soy bean extract. Migration time increases with increasing pH. The addition of boric acid to the background electrolyte increases the retention time of each isoflavone because each isoflavone reacts with the boric acid to form negatively charged borate complexes. Both the migration time for each flavonoid and the overall resolution of the separation increases with increasing concentrations of boric acid. Pietta et al. (1994) studied the influence of the structure on the behavior of flavonoids in CZE. The effect of

pH, surfactant, and buffer on the resolution several flavonoids differing in structure has been investigated. Substitution of ring B and the presence of a free hydroxyl group at C7 of ring A plays an important role, whereas *O*-methylation is less significant. Glycosylation of the flavonoid also contributes in determining different degrees of complexation with borate, thus affecting the mobilities. The influence of pH and structure on the electrophoretic mobility of flavonoids is discussed in McGhie and Makham (1994, 1996).

A capillary electrophoretic method has been developed to analyze, simultaneously, citrus juice components in a single procedure (Cancalon, 1999). Compounds regularly monitored include the flavonoids didymin, hesperidin, narirutin, neohesperidin, and naringin. This method allows rapid monitoring of citrus juices, giving information on quality, freshness, and possible adulteration of the product, and could be used to monitor other fruit juices and quantitate diverse blends.

9.4.6 MICELLAR ELECTROKINETIC CHROMATOGRAPHY

The main strength of micellar electrokinetic chromatography (MEKC) is that it is the only electrophoretic technique that can be used for the separation of neutral solutes as well as charged ones. The separation of neutral species is accomplished by using surfactants in the running buffer. Partitioning in and out of the micelles formed in the surfactant affects the separation. MEKC has been successfully used for separation of phenolic compounds (Rodriguez-Delgado et al., 2000, 2002). The influence of buffer, concentration of surfactant, and applied voltage was studied. Organic solvent was added to the separation buffer to increase the resolution. This method was applied to the determination of flavonoids in wine samples with good results.

9.5 CONCLUSION

Flavonoids have undoubtedly shown *in vitro* benefits to human health. More *in vivo* studies are needed to ascertain the beneficial effects of flavonoids and to see whether there are any dangers in possible overdoses, especially in the form of supplements. Though capillary electrophoresis is beginning to be used for the analysis of flavonoids, HPLC by far is the method of choice, with several hundred papers published in the last two decades. Since antioxidant activity is associated with flavonoids, HPLC with coulometric array detection might shed more light on the analysis of flavonoids in the future.

REFERENCES

- Amarowicz, R. and Shahidi, F., A rapid chromatographic method for separation of individual catechins from green tea, *Food Res. Int.*, 29, 71–76, 1996.
- Arpino, P., Combined liquid chromatography-mass spectrometry. I, *Mass Spectrom. Rev.*, 8, 35–55, 1989.
- Arpino, P., Combined liquid chromatography-mass spectrometry. II, *Mass Spectrom. Rev.*, 9, 631–669, 1990.

- Arts, I.C.W. and Hollman, P.C.H., Optimization of a quantitative method for the determination of catechins in fruits and legumes, *J. Agric. Food Chem.*, 46, 5156–5162, 1998.
- Aussenac, T., Lacombe, S., and Dayde, J., Quantification of isoflavones by capillary zone electrophoresis in soybean seeds: effects of variety and environment. *Am. J. Clin. Nutr.*, 68, 1480S–1485S, 1998.
- Bakker, J., Bridle, P., and Koopman, A., Strawberry juice color: the effect of some processing variables on the stability of anthocyanins, *J. Sci. Food Agric.*, 60, 471–476., 1992
- Bingham, S.A., Atkinson, C., Liggins, J., Bluck, L., and Coward, A., Phyto-oestrogens: where are we now? *Br. J. Nutr.*, 79, 393–406, 1998.
- Bloor, S.J., Overview of methods for analysis and identification of flavonoids, *Methods Enzymol.*, 335, 3–14, 2001.
- Bogdanov, S., Determination of pinocembrin in honey using HPLC, *J. Apic. Res.*, 28, 55–57, 1989.
- Bronner, W.E. and Beecher, G.R., Method for determining the content of catechins in tea infusions by high-performance liquid chromatography, *J. Chromatogr. A*, 805, 137–142, 1998.
- Canalón, P.F., Analytical monitoring of citrus juices by using capillary electrophoresis, *J. AOAC Int.*, 82(1), 95–106, 1999.
- Canalón, P.F. and Bryan, C.R., Use of capillary electrophoresis for monitoring citrus juice composition, *J. Chromatogr. A*, 652, 555–561, 1993.
- Carbonell, S.A., Aquino Neto, F.R., Cardoso, J.N., Pereira, A.S., Amaral, A.C.F., and Barnes, B.A., Rapid screening of natural products by high-resolution high-temperature gas chromatography, *J. Chromatogr. Sci.*, 38, 234–239, 2000.
- Daigle, D.J. and Conkerton, E., Analysis of flavonoids by HPLC, *J. Liq. Chromatogr.*, 6, 105–118, 1983.
- Daigle, D.J. and Conkerton, E.J., Analysis of flavonoids by HPLC: an update, *J. Liq. Chromatogr.*, 11, 309–325, 1988.
- Dalluge, J.J., Nelson, B.C., Thomas, J.B., and Sander, L.C., Selection of column and gradient elution system for the separation of catechins in green tea using high-performance liquid chromatography, *J. Chromatogr. A*, 793, 265–274, 1998.
- de Pascual-Teresa, S., Treutter, D., Rivas-Gonzalo, J.C., and Santos-Buelga, C., Analysis of flavonols in beverages by high-performance liquid chromatography with chemical reaction detection, *J. Agric. Food Chem.*, 46, 4209–4213, 1998.
- Donner, H., Gao, L., and Mazza, G., Separation and characterization of simple malonylated anthocyanins in red onions, *Allium cepa* L., *Food Res. Int.*, 30, 637–643, 1997.
- Finger, A., Engelhardt, U.H., and Wray, V., Flavonol triglycosides containing galactose in tea, *Phytochemistry*, 30, 2057–2060, 1991a.
- Finger, A., Engelhardt, U.H., and Wray, V., Flavonol glycosides in tea-kaempferol and quercetin rhamnoglucosides, *J. Sci. Food Agric.*, 55, 313–321, 1991b.
- Formica, J.V. and Regelson, W., Review of the biology of Quercetin and related bioflavonoids, *Food Chem. Toxicol.*, 33, 1061–1080, 1995.
- Gao, L. and Mazza, G., Rapid method for complete characterization of simple and acylated anthocyanins by high-performance liquid chromatography and capillary gas-liquid chromatography, *J. Food Agric. Food Chem.*, 42, 118–125, 1994.
- Gao, L. and Mazza, G., Characterization, quantitation, and distribution of anthocyanins and colorless phenolics in sweet cherries, *J. Agric. Food Chem.*, 43, 343–346, 1995.
- Gil, M.I., Cherif, J., Ayed, N., Artes, F., and Tomas-Barberan, F., Influence of cultivar, maturity stage and geographical location on the juice pigmentation of Tunisian pomegranate, *Z. Lebensm. Unters. Forsch.*, 201, 361–364, 1995a.

- Gil, M.I., Garcia-Viguera, C., Artes, F., and Tomas-Barberan, F.A., Changes in pomegranate juice pigmentation during ripening, *J. Sci. Food Agric.*, 68, 77–81, 1995b.
- Goiffon, J.P., Brun, M., and Bourrier, M.J., High performance liquid chromatography of red fruit anthocyanins, *J. Chromatogr.*, 537, 101–121, 1991.
- Goldberg, D.M., Karumanchiri, A., Tsang, E., and Soleas, G.J., Catechin and epicatechin concentration of red wines: regional and cultivar-related differences, *Am. J. Enol. Vitic.*, 49, 23–34, 1998.
- Guyot, S., Marnet, N., Laraba, D., Sanoner, P., and Drilleau, J.F., Reversed-phase HPLC following thiolysis for quantitative estimation and characterization of the four main classes of phenolic compounds in different tissue zones of a French cider apple variety (*Malus domestica* var. *Kermesrien*), *J. Agric. Food Chem.*, 46, 1698–1705, 1998.
- Harborne, J.B. and Grayer, R.J., Flavonoids and insects, in *The Flavonoids*, Harborne, J.B., Ed., Chapman & Hall, London, 1994.
- Hasegawa, S., Berhow, M.A., and Fong, C.H., Analysis of bitter principles in citrus, *Mod. Methods Plant Anal.*, 18, 59–80, 1996.
- Hertog, M.G.L., Hollman, P.C., and Venema, D.P., Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruit, *J. Agric. Food Chem.*, 40, 1591–1598, 1992.
- Hong, V. and Wrolstad, R.E., Use of HPLC separation/photodiode array detection for characterization of anthocyanins, *J. Agric. Food Chem.*, 38, 708–715, 1990.
- Hutabarat, L.S., Mulholland, M., and Greenfield, H., Development and validation of an isocratic high-performance liquid chromatographic method for quantitative determination of phytoestrogens in soya bean, *J. Chromatogr.*, 795, 377–382, 1998.
- Issaq, H.J., Capillary electrophoresis of natural products. II, *Electrophoresis*, 20, 3190–3202, 1999.
- Justesen, U., Knuthsen, P., and Leth, T., Quantitative analysis of flavonols, flavones, and flavanones in fruits, vegetables and beverages by high-performance liquid chromatography with photo-diode array and mass spectrometric detection, *J. Chromatogr. A*, 799, 101–110, 1998.
- Khokhar, S., Venema, D., Hollman, P.C., Dekker, M., and Jongen, W., A RP-HPLC method for the determination of tea catechins, *Cancer Lett.*, 114, 171–172, 1997.
- Krause, M. and Galensa, R., Improved chiral stationary phase based on cellulose triacetate supported in a non-macroporous silica gel diol for the high-performance liquid chromatographic separation of racemic flavonones and diastereomeric flavanone glycosides, *J. Chromatogr.*, 502, 287–296, 1990.
- Liang, Y.R., Liu, Z.S., Xu, Y.R., and Hu, Y.L., A study on chemical composition of two special green teas (*Camellia sinensis*). *J. Sci. Food Agric.*, 53, 541–548, 1990.
- Lin, Y.Y., Ng, K.J., and Yang, S., Characterization of flavonoids by liquid chromatography, *J. Chromatogr.*, 629, 389–393, 1993.
- Mabry, T.J., Markham, K.R., and Thomas, M.B., *The Systematic Identification of Flavonoids*, Springer-Verlag, New York, 1970.
- Marini, D. and Balestrieri, F., Multivariate analysis of flavanone glycosides in citrus juices, *Ital. J. Food Sci.*, 7, 255–264, 1995.
- Mauri, P.L., Iemoli, L., Gardana, C., Riso, P., Simonetti, P., Porrini, M., and Pietta, P.G., Liquid chromatography/electrospray ionization mass spectrometric characterization of flavonol glycosides in tomato extracts and human plasma, *Rapid Commun. Mass Spectrom.*, 13, 924–931, 1999.
- Mazur, V.M., Duke, J.A., Wähälä, K., Rasku, S., and Adlercreutz, H., Isoflavonoids and lignans in legumes: nutritional and health aspects in humans, *Nutr. Biochem.*, 9, 193–200, 1998.

- Markham, K.R. and Bloor, S.J., Analysis and identification of flavonoids in practice, in *Flavonoids in Health and Disease*, Rice-Evans, C.A. and Packer, L., Eds., Marcel Dekker, New York, 1998, pp. 1–34.
- Markham, K.R. and McGhie, T.K., Separation of flavones by capillary electrophoresis; the influence of pK_a on electrophoretic mobility, *Phytochem. Anal.*, 7, 300–304, 1996.
- McGhie, T.K., Analysis of sugar cane flavonoids by capillary zone electrophoresis, *J. Chromatogr.*, 634, 107–112, 1993.
- McGhie, T.K. and Markham, K.R., Separation of flavonols by capillary electrophoresis: the effect of structure on electrophoretic mobility, *Phytochem. Anal.*, 5, 121–126, 1994.
- Merken, H.M. and Beecher, G.R., Measurements of food flavonoids by high performance liquid chromatography: a review, *J. Agric. Food Chem.*, 48, 3, 577–599, 2000a.
- Merken, H.M. and Beecher, G.R., Liquid chromatographic method for the separation and quantification of prominent flavonoid aglycones, *J. Chromatogr.*, 897, 177–184, 2000b.
- Milbury, P.E., Analysis of complex mixtures of flavonoids and polyphenols by high-performance liquid chromatography electrochemical detection methods, in *Methods Enzymol.*, 335, 15–26, 2001.
- Mouly, P., Gaydou, E.M., and Estienne, J., Column liquid chromatographic determination of flavanone glycosides in citrus. Application to grapefruit and sour orange adulteration, *J. Chromatogr.*, 634, 129–134, 1993.
- Mouly, P.P., Argouyan, C.R., Gaydou, E.M., and Estienne, J.M., Differentiation of citrus juices by factorial, discriminant analysis using liquid chromatography of flavanone glycosides, *J. Agric. Food Chem.*, 42, 70–79, 1994.
- Niessen, W.M.A., Advances in instrumentation in liquid chromatography-mass spectrometry and related liquid-introduction techniques, *J. Chromatogr.*, 794A, 407–435, 1998.
- Ogawa, A., Arai, H., Taniuzawa, H., Miyahara, T., and Toto'oka, T., On-line screening method for antioxidants by liquid chromatography with chemiluminescence detection, *Anal. Chim. Acta*, 383, 221–230, 1999.
- Pietta, P., Mauri, P., Bruno, A., and Gardana, C., Influence of structure on the behavior of flavonoids in capillary electrophoresis, *Electrophoresis*, 15, 1326–1331, 1994.
- Rice-Evans, C.A., Miller, N.J., and Paganga, G., Structure-antioxidant activity relationships of flavonoids and phenolic acids, *Free Radic. Biol. Med.*, 20, 933–956, 1996.
- Robards, K. and Antolovich, M., Analytical chemistry of fruit bioflavonoids, *Analyst*, 122, 11R–34R, 1997.
- Rodriguez, D., Perez, M.L., Corbella, R., Gonzalez, G., and Garcia Montelongo, F.J., Optimization of the separation of phenolic compounds by micellar electrokinetic capillary chromatography, *J. Chromatogr. A*, 871, 427–438, 2000.
- Rodriguez-Degado, M.A., Perez, M.L., Corbella, R., Gonzalez, G., and Garcia-Montelongo, F.J., Optimization of the separation of phenolic compounds by micellar electrokinetic capillary chromatography, *J. Chromatogr.*, 87(1–2), 427–438, 2000.
- Rodriguez-Delgado, M.A., Garcia-Montelongo, F.J., and Cifuentes, A., Ultrafast sodium dodecyl sulfate micellar electrokinetic chromatography with very acidic running buffers, *Anal. Chem.*, 74(1), 257–260, 2002.
- Rodriguez-Saona, L.E., Gousti, M., and Wrolstad, R.E., Anthocyanin pigment composition of red-fleshed potatoes, *J. Food Sci.*, 63, 458–465, 1998.
- Rovellini, P., Cortesi, N., and Fedeli, E., Analysis of flavonoids from *Olea europea* by HPLC-UV and HPLC-electrospray-MS, *Riv. Ital. Sostanze Grasse*, 74, 273–279, 1997.
- Samuelsson, G., *Drugs of Natural Origin, A Textbook of Pharmacognosy*, 4th ed., Swedish Pharmaceutical Press, 1999, pp. 226–227.

- Stenner, W., et al., Comparison of high-performance chromatography, supercritical fluid chromatography and capillary zone electrophoresis in drug analysis, *J. Chromatogr.*, 507, 125–140, 1990.
- Stobiecki, M., Application of mass spectrometry for identification and structural studies of flavonoid glycosides, *Phytochemistry*, 54, 237–256, 2000.
- Strack, D. and Wray, V., The anthocyanins, in *The Flavonoids*, Harborne, J.B., Ed., Chapman & Hall, London, 1994.
- Takeoka, G.R., Dao, L.T., Full, G.H., Wong, R.Y., Harden, L.A., Edwards, R.H., and Berrios, J.D., Characterization of black bean (*Phaseolus vulgaris* L.) anthocyanins, *J. Agric. Food Chem.*, 45, 3995–4000, 1997.
- Wang, J. and Sporns, P., Analysis of anthocyanins in red wine and fruit juice using MALDI-MS, *J. Agric. Food Chem.*, 47, 2009–2015, 1999.
- Wang, J. and Sporns, P., MALDI-TOF MS analysis of food flavonol glycosides, *J. Agric. Food Chem.*, 48(5), 1657–1662, 2000.

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