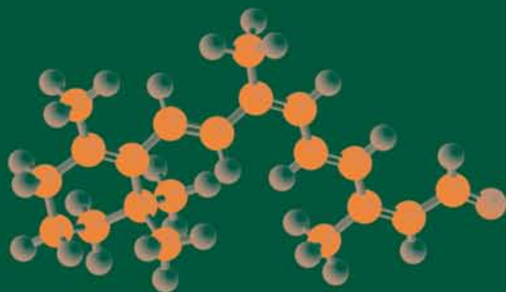
Second Edition

VITAMIN ANALYSIS FOR THE HEALTH AND FOOD SCIENCES



Ronald R. Eitenmiller
Lin Ye
W. O. Landen, Jr.

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FOR THE HEALTH
AND FOOD SCIENCES
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Preface

During our careers at the University of Georgia and with the U.S. Food and Drug Administration, we (Eitemiller and Landen) have been privileged to experience and participate in the rapid advancement of vitamin assay methodology. From the early 1970s, this field progressed from reliance on techniques developed decades earlier to a degree of sophistication that few of us foresaw. Indeed, we clearly remember the effort required to obtain valid data on the vitamin content of the food supply using microbiological, thin-layer, and open-column chromatographic and spectrophotometric assays. We know that scientists investigating the vitamins in clinical samples had similar experiences. A great deal of discipline was required on the part of experienced analysts and graduate students to produce analytical values, which, for the most part, have withstood the test of time when compared with values obtained with current, much improved, and sophisticated instrumentation.

Challenges, we firmly appreciate, are the training of students and analysts in the proper application of the best vitamin assay methods and the frequently required efforts to improve, develop, or adapt existing methods to meet specific analytical needs. The first edition, published in 1999, was written as a source for these activities. We were pleased with the good reception of the first edition on an international level.

Our discussion on each vitamin includes a review section aimed at providing individuals who have not studied the vitamins in depth an appreciation of the uniqueness of the vitamin and how it participates in metabolism. This section has purposefully been kept brief. However, the references provided can be highly useful for those seeking additional information.

We strongly feel that individuals involved in the analysis of any analyte must have a basic understanding of the chemistry of the compound. Therefore, chemistry and nomenclature of each vitamin is discussed. The information might be considered inadequate by the research biochemist or analytical chemist involved in basic studies on a specific vitamin; however, our goal was to produce a usable source for analysts at the bench and not to write a multivolume treatise. Extensive information is given in tabular form on spectral properties. Such data is routinely required on a day-to-day basis at the bench. In addition, stability properties of the vitamins are discussed. Stability considerations can be easily disregarded or forgotten during routine analysis or during method development if the chemist is not thoroughly aware of the specific properties of the vitamin that affect stability. If this happens, extensive efforts can be negated when the oversight is recognized.

In the methods section of each chapter, our purpose was twofold. First, we felt that attention needed to be given to commonly used and available handbook and regulatory methods. These accepted procedures are in use across the world and, from a regulatory standpoint, maintain significant status. A summary table is provided covering many of

these sources. In addition, several of the Association of Official Analytical Chemists (AOAC) International methods are discussed in detail. Second, our primary objective was to give an interpretive review of the development of advanced methods of vitamin analysis in sufficient detail to be valuable as a methodology guide.

We (Eitenmiller and Landen) were fortunate to include Dr. Lin Ye as an author for the second edition. Dr. Ye adds significant expertise to pharmaceutical and food analysis from an industrial and method development aspect. Most importantly, Dr. Ye brought insight into the rapidly developing use of liquid chromatography-mass spectrometry (LC-MS) for food and drug analysis. Indeed, LC-MS is at the same point of rapid acceptance as the primary and best tool for vitamin assay, similar to the acceptance of LC in the mid-1970s. We have tried to show the most recent applications and impact of LC-MS on vitamin analysis throughout the second edition.

When analysis programs are being initiated, much effort can be saved in making correct decisions about analytical approach if the analyst has a thorough grasp of research leading to available methods. For the vitamins for which LC is the best approach, detailed tables are presented, describing historically significant method development advances that led the way to current methods as well as significant publications, which appeared in 2007. It is evident that, for each vitamin, the efforts of a few research groups have driven the field to its current capability to accurately assay the vitamin. We hope that our discussion has given the credit where it is due. At the same time, the literature is voluminous and expanding extremely rapidly. It is impossible to cite all efforts playing a role in vitamin analysis. If this book can lead those endeavoring to initiate a vitamin analysis program to the right groups of investigators producing the current advances, then we have accomplished our purpose to provide a usable source for today's vitamin chemist.

Ronald R. Eitenmiller
Lin Ye
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Dr. Lin Ye is a research scientist at the Coca-Cola Company, Atlanta, Georgia. Her current research focus is the development and validation of liquid chromatography and mass spectroscopy (LC-MS/MS) methods for analysis of food ingredients. Dr. Ye received her PhD in food science at the University of Georgia in 1999 and has published widely. She has more than 20 years of industrial and academic experience in the development of LC, LC-MS, LC-MS/MS, and gas chromatography methods for food and drug analysis.

W. O. Landen, Jr. retired from the U.S. Food and Drug Administration (FDA). He was a research supervisory chemist with the Atlanta Center for Nutrient Analysis. His work with the FDA was recognized by ten outstanding or special performance awards. He has served on the Association of Official Analytical Chemists (AOAC) International Methods Committee on Food and Nutrition and on the AOAC-NLEA Task Force on Nutrient Analysis Methods. Landen continues to consult with the food and pharmaceutical industries. He has published widely on analytical method development throughout his career.

List of Abbreviations

A	Acetone
Å	Angstrom
AA	Ascorbic acid
AC	Activity Coefficient
ACNA	Atlanta Center for Nutrient Analysis
ACP	Acyl-carrier protein
AdoCbl	Deoxyadenosyl cobalamin
AlAT	Alanine Aminotransferase
AMP	Ascorbate-2-monophosphate
AMS	Ascorbate-2-monosulfate
AOAC	Association of Official Analytical Chemists
APDC	Ammonium pyrrolidinedithiocarbamate
APP	Ascorbate polyphosphate
AsAT	Aspartate Aminotransferase
B	Benzene
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BSC	Benzenesulfonyl chloride
BTA	1,3,5-Benzene-tricarboxylic acid
BTP	Bis-*tris*-propane
$C_2H_4Cl_2$	1,2-Dichloroethane
CDRF	Cyclodehydroriboflavin
CE	Capillary electrophorosis
CH_2Cl_2	Dichloromethane
$CHCl_3$	Chloroform
CI	Chemical ionization
CIS	Coordination ion spray
cm	Centimeter
CNCbl	Cyanocobalamin
CPBA	Competitive Protein binding assays
CV	Coefficient of variation
DCI	Desorption chemical ionization
DCIP	2,6-Dichloroindophenol
DHAA	Dehydroascorbic acid
DIEA	*N*,*N*-diisopropylethylamine
DIPE	Diisopropylether

DL	Detection limit
DMEQ-TAD	4-[2-(6,7-Dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxaly)ethyl]-1,2,4-triazoline-3,5-dione
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNPH	2,4-Dinitrophenylhydrazine
DTMAC	Dodecyltrimethyl ammonium chloride
dTMP	Thymidylate
DTT	Dithiothreitol
e	Molar absorptivity
$E^{1\%}_{1cm}$	Specific extinction coefficient
EC	Electrochemical
ECNI	Electron capture negative ionization
EGR	Erythrocyte glutathione reductase
EI	Electron impact
ELISA	Enzyme-linked immunosorbent assay
ELSD	Evaporative light scattering detector
Em	Emission
EPBA	Enzyme protein-binding assay
ESI	Electrospray ionization
Et_2O	Diethyl ether
EtOAc	Ethyl acetate
EtOH	Ethyl alcohol
Ex	Excitation
FAD	Flavin adenine dinucleotide
FIA	Flow-injection analysis
FID	Flame ionization detection
FIGLU	Formiminoglutamic acid
FITC	Fluorescein isothiocyanate
FMN	Flavin mononucleotide
fmol	Femtomol
g	Gram
GLC	Gas liquid chromatography
h	Hours
H_2OCbl	Aquocobalamin
HAC	Acetic acid
HCI	Hydrochloric acid
HDTMAB	Hexadecyl trimethylammonium bromide
Hep	Heptane
Hex	Hexane
HFBA	Heptafluorobutyric acid
HPCE	High Performance Capillary Electrophoresis
HP-GPC	High performance-gel permeation chromatography
HPLC	High performance liquid chromatography
HSA	Sodium hexanesulphonate
HTST	High Temperature Short Time
IAA	Isoascorbic acid
iOCT	Isooctane
IPA	Isopropyl alcohol
IS	Internal standard

IU	International unit
KCN	Potassium cyanide
kg	Kilogram
L	Liter
LC	Liquid chromatography
LTLT	Low Temperature Long Time
M	Molar
2-MCE	2-Mercaptoethanol
MeCbl	Methylcobalamin
MeCN	Acetonitrile
MEKC	Micellar electrokinetic chromatography
MeOH	Methanol
mL	Milliliter
mm	Millimeter
mM	Millimolar
mmol	Millimole
1-MP	1-Methylpiperidine
MPA	Metaphosphoric acid
MPB	Menadione dimethyl pyrimidinol bisulfate
MPCSC	6-Methyl-2-pyridine carboxaldehyde semicarbazine
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MSB	Menadione sodium bisulfate
MSBC	Menadione sodium bisulfate complex
MTBE	Methyl-*tert*-butyl ether
mV	Millivolt
N	Normal
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NaOAc	Sodium acetate
NE	Niacin equivalent
NH_4OAc	Ammonium acetate
NIST	National Institute of Standards and Technology
NLEA	Nutritional Labeling and Education Act of 1990
nm	Nanometer
nM	Nanomol
NMN	*N*-Methylnicotinamide
nmol	Nanomole
NMR	Nuclear magnetic resonance
NO_2Cbl	Nitrocobalamin
NP	Normal Phase
ODS	Octyldecylsilica
OHCbl	Hydroxycobalamin
OPD	Orthophenyldiamine (*O*-phenylenediamine)
OTE	Dithioerythritol
OTMAB	Dodecyltrimethyl ammonium bromide
4-PA	4-Pyridoxic acid
PABG	*p*-Aminobenzoyl glutamic acid
PDA	Photodiode array
PE	Petroleum ether

pg	Picogram
PL	Pyridoxal
PLP	Pyridoxal-5′-phosphate
PM	Pyridoxamine
pM	Picomol
PMC	2,2,5,7,8-Pentamethyl-6-hydroxy chroman
PMP	Pyridoxamine-5′-phosphate
PN	Pyridoxine (pyridoxol)
PN-glucoside	Pyridoxine-glucoside
PN-HC1	Pyridoxine HC1
PNP	Pyridoxine-5′-phosphate
PPB	Parts per billion
Pt	Platinum
PT	Prothrombin time
PUFA	Polyunsaturated fatty acid
QL	Quantitation limit
rac	Racemic
RCM	Radial compression module
RDA	Recommended Dietary Allowance
RDI	Reference Daily Intake
RE	Retinol equivalent
RIA	Radioimmunoassay
RP	Reversed-phase
RRA	Radioreceptor assay
RSD	Relative standard deviation
Sep. funnel	Separatory funnel
SIM	Selected ion monitoring
SO_3Cbl	Sulfitocobalamin
SPE	Solid-phrase extraction
SRM	Selected reaction monitoring
SSA	Sulfosalicylic acid
T	Toluene
TBAB	Tetrabutylammonium bromide
TBAH	*tert*-Butylammonium hydroxide
TBAHS	Tetrabutylammonium hydrogen sulfate
TBAP	Tetrabutylammonium phosphate
TBME	*tert*-Butyl methyl ether
TCA	Trichloroacetic acid
TCA cycle	Tricarboxylic acid cycle
TEA	Triethylamine
TFA	Trifluoroacetic acid
ThcMP	Thiachrome monophosphate
ThcPP	Thiachrome pyrophosphate
ThcTP	Thiachrome triphosphate
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMP	Thiamin monophosphate
TMS	Trimethyl-silyl
TOAB	Tetraoctylammonium bromide
TPP	Thiamin pyrophosphate

TSP	Thermospray
TTP	Thiamin triphosphate
UL	Tolerable upper intake level
USP	United States Pharmacopeial Convention
UV	Ultraviolet
V	Volts
Vitamin D_2	Ergocalciferol
Vitamin D_3	Cholecalciferol
α-T	α-Tocopherol
α-T3	α-Tocotrienol
β-T	β-Tocopherol
β-T3	β-Tocotrienol
γ-T	γ-Tocopherol
γ-T3	γ-Tocotrienol
δ-T	δ-Tocopherol
δ-T3	δ-Tocotrienol
µg	Microgram
µL	Microliter
µm	Micrometer

section one

Fat-soluble vitamins

chapter one

Vitamin A and carotenoids

1.1 Review

The relationship of night blindness to a dietary deficiency was recognized as early as 1500 BC.[1] In 1913, McCollum and Davis reported the presence of a lipid-like substance in butter and egg yolk that was necessary for growth in rats. In 1916, the substance was named fat-soluble A. McCollum related fat-soluble A deficiency to xerophthalmia in children in the following year, providing the first indication of the diverse functionality of the vitamin. The name, vitamin A, was first used in 1920 to signify the early discovery of the growth factor and to differentiate it from water-soluble vitamins, collectively called vitamin B at that time. The structure of vitamin A was determined in 1931. The vitamin A activity of β-carotene was demonstrated in 1929. The term "provitamin A" is accepted to differentiate carotenoid precursors of vitamin A from carotenoids without vitamin A activity. Plant carotenoids are, therefore, the precursor of vitamin A found in the animal kingdom. Dietary vitamin A is designated 'preformed vitamin A' when consumed as a dietary constituent of animal products.

Vitamin A deficiency is characterized by changes in the tissue of the eye that ultimately result in irreversible blindness. Clinical symptoms are collectively referred to as xerophthalmia[2] and include the following:

1. Night blindness—the inability to see in dim light
2. Conjunctival and corneal xerosis
3. Keratomalacia—ulceration and scarring of the cornea that leads to loss of vision

Other symptoms include skin lesions, loss of appetite, epithelial keratinization, lack of growth, and increased susceptibility to infections.[2–4] Human status assessment methods include dietary assessment, assessment of content of liver, plasma, and breast milk, and functional assessment by dark adaptation and conjunctival impression cytology.[4] Biochemical tests include the relative dose response test (RDR) and the modified relative dose response test (MRDR) that estimate liver stores of vitamin A. Such tests are useful to assess marginal vitamin A deficiency.[5] Plasma retinol concentration is a commonly used measure of status.[2,5] Measurement of the concentration of retinol binding protein in plasma is becoming an accepted status measure for clinical studies. However, the relationship of the retinol-binding protein concentration to the accepted retinol concentration of $<0.70\ \mu mol\ L^{-1}$ that reflects inadequacy is not completely defined.[5] More recent clinical studies of deficiency use serum retinol concentration as the status indicator.[6,7]

Vitamin A toxicity (hypervitaminosis A) can occur because of high intake from either food high in vitamin A or high-potency supplements. Toxicity to retinoids has been

classified as acute, chronic, and teratogenic.[2,8,9] Acute toxicity results from a single dose or a limited number of large doses over a short time period. A single dose greater than 200 mg (>200,000 retinol activity equivalent [RAE] >660,000 IU or 0.7 mmol) or all-*trans*-retinol can result in acute toxicity in adults. For children, 50% of the adult dose can cause acute toxicity.[8] Symptoms include nausea, vomiting, headache, increased cerebrospinal fluid pressure, vertigo, blurred vision, muscle incoordination, and bulgind fonanel in infants.[2] Chronic toxicity results from ingestion of large doses at or above 30 mg (30,000 RAE) per day for months or years.[2] Symptoms of chronic toxicity include alopecia, ataxia, liver abnormalities, membrane dryness, bone and skin changes, visual impairment, and nervous system effects.[2,8] Most symptoms are reversible when vitamin A intake is decreased.[8] Teratogenic effects can result from simple, large doses (30–90 mg) or long-term intakes that result in chronic toxicity.[8] Common teratogenic defects include physical malformations, heart, kidney, and thymic disorders, and central nervous system disorders.[8] Penniston and Tanumihardjo[9] show in a recent review that new studies suggest that subtoxicity without clinical signs of toxicity may be problematic in developed countries. Intake from preformed sources often exceeds recommended dietary allowances (RDAs). As an example of such concern, osteoporosis and hip fracture are associated with retinol intakes only twice the RDA.[9]

Dietary sources of vitamin A include organ meats (the liver contains the highest amount), fish oils, butter, eggs, whole milk and fortified low fat milk, other dairy products, and fish (particularly fish with higher fat content like tuna and sardines). Margarine, fluid milk, and dry milk are typically fortified with retinyl palmitate in many countries. These products play a dramatic role in preventing vitamin A deficiency in countries where fortification is mandatory. In the United States, milk is fortified with not less than 2,000 IU of vitamin A (retinyl palmitate). Margarine is fortified with not less than 15,000 IU per pound (all-*trans*-retinyl palmitate and β-carotene).[10] Data from the USDA National Nutrient Database on food content is given in Table 1.1.[11]

Provitamin A carotenoids are found throughout the plant kingdom. Food sources include carrots, dark-green leafy vegetables, and various fruits. Because of the complexity of the carotenoid profiles of fruits and vegetables, color cannot be used to predict the vitamin A activity of the food. Lycopene is frequently used to demonstrate this point. The red color of ripe tomatoes is primarily due to this carotenoid that possesses no vitamin A activity. Thus, most tomato cultivars are quite low in vitamin A activity. Approximately 75% of the vitamin A in the U.S. diet is derived from retinol and 25% from provitamin A carotenoids.[12] A USDA/NCI database containing compositional information on α-carotene, β-carotene, β-cryptoxanthin, lutein plus zeaxanthin, and lycopene has been compiled for over 2400 fruits, vegetables, and foods containing fruits and vegetables.[13–15] This database provides a valuable tool for determining dietary intake of the carotenoids.[16]

Before the publication of the Dietary Reference Intakes (DRIs)[2] the biological activity of vitamin A was quantified by conversion of the vitamin A active components to retinol equivalents (RE). One RE is defined as 1 μg of all-*trans*-retinol. For calculation of RE values in foods, 100% efficiency of absorption of all-*trans*-retinol is assumed. Incomplete absorption and conversion of β-carotene is taken into account by the relationship of 1 RE = 6 μg of β-carotene. Other provitamin A active carotenoids have approximately 50% of the biological activity of β-carotene. The conversion factor is, therefore, 1 RE = 12 μg of other provitamin A carotenoids. The RDA was 1000 RE for adult men and 800 RE for adult women.

On the basis of newer information on absorption of dietary carotenoids, the Institute of Medicine[2] established the microgram retinol activity equivalent (μg RAE) value to replace the RE as a measure of the vitamin A acitivity of dietary provitamin A carotenoids. One RAE is defined as 1 μg of all-*trans*-retinol, 12 μg of all-*trans*-β-carotene, and 24 μg of other provitamin A carotenoids (usually limited to α-carotene and β-cryptoxanthin).

Table 1.1 Vitamin A Activity of Various Foods

Description	NDB No	µg RAE[a] 100 g^{-1}	Description	NDB No	µg RAE[a] 100 g^{-1}
Turkey, all classes, giblets, cooked, simmered, some giblet fat	05172	10737	Beet greens, cooked, boiled, drained, without salt	11087	383
Beef, variety meats and by-products, liver, cooked, pan-fried	13327	7744	Turnip greens, cooked, boiled, drained, without salt	11569	381
Braunschweiger (a liver sausage), pork	07014	4221	Lettuce, green leaf, raw	11253	370
Malted drink mix, chocolate, with added nutrients, powder	14315	3929	Cheese, cream	01017	366
Chicken, broilers or fryers, giblets, cooked, simmered	05022	1753	Mustard greens, cooked, boiled, drained, without salt	11271	316
Sweet potato, cooked, baked in skin, without salt	11508	961	Squash, winter, all varieties, cooked, baked, without salt	11644	261
Carrot juice, canned	11655	956	Cereals ready-to-eat, KELLOGG, KELLOGG'S RAISIN BRAN	08060	254
Carrots, frozen, cooked, boiled, drained, without salt	11125	860	Cabbage, chinese (pak-choi), cooked, boiled, drained, without salt	11117	212
Carrots, raw	11124	841	Egg, whole, cooked, fried	01128	198
Margarine, regular, tub composite, 80% fat, with salt	04611	817	Melons, cantaloupe, raw	09181	169
Sweet potato, cooked, boiled, without skin	11510	787	Lettuce, butterhead (includes boston and bibb types), raw	11250	166
Pumpkin, canned, without salt	11424	778	Egg, whole, raw, fresh	01123	140
Kale, frozen, cooked, boiled, drained, without salt	11236	735	Cheese, ricotta, whole milk	01036	120
Butter, salted	01001	683	Milk, canned, evaporated, nonfat	01097	118
Spinach, frozen, chopped or leaf, cooked, boiled, drained, without salt	11464	603	Milk, canned, condensed, sweetened	01095	74
Vegetables, mixed, canned, drained solids	11581	582	Milk, nonfat, fluid, with added vitamin A (fat free or skim)	01085	61
Cereals ready-to-eat, KELLOGG, KELLOGG'S FROSTED FLAKES	08069	516	Milk, chocolate, fluid, commercial, lowfat	01104	58
Cereals ready-to-eat, GENERAL MILLS, KIX	08048	507	Milk, reduced fat, fluid, 2% milk fat, with added vitamin A	01079	55
Cereals ready-to-eat, GENERAL MILLS, CHEERIOS	08013	500	Mangoes, raw	09176	38
Cereals ready-to-eat, GENERAL MILLS, WHEATIES	08089	500	Cheese, cottage, creamed, with fruit	01013	38
Spinach, raw	11457	470	Soy milk, fluid	16120	31
Collards, cooked, boiled, drained, without salt	11162	406	Watermelon, raw	09326	28
Sweet potato, canned, vacuum pack	11512	399	Milk, whole, 3.25% milkfat	01077	28

[a] µg RAE: µg retinol activity equivalents.

Source: United States Department of Agriculture, Agricultural Research Service, 2006, USDA Nutrient Database for Standard Reference, Release 19, Nutrient Data Laboratory Home Page, http://www.nal.usda.gov/fnic/foodcomp, Riverdale, MD: Nutrient Data Laboratory, USDA.

International units (IUs) are used for labeling pharmaceuticals, supplements, and foods. International units (IU) are defined by the relationship one IU equals 0.3 μg of all-*trans*-retinol or 0.6 μg of β-carotene. Therefore, 1 μg of all-*trans*-retinol equals 3.33 IU of vitamin A activity. Original studies that estimated the vitamin A activity of the carotenoids and defined IU did not account for bioavailability differences as compared to all-*trans*-retinol.[2] The Reference daily intake (RDI) set by the Nutritional Labeling and Education Act of 1990 (NLEA) is 5000 IU.[17]

Establishment of the RAE was based on the accepted carotene:retinol equivalency ratio (μg) of a low dose of purified β-carotene in oil of 2:1, indicating that 2 μg of β-carotene in oil yields 1 μg of retinol.[2] Differences between RE conversion factors and RAE factors stem from absorption studies that show that 6 μg of dietary β-carotene is equal to 1 μg of β-carotene in oil ([6 × 2]:1 = 12:1); the RAE calculation doubles the amount of dietary provitamin A carotenoids required to provide 1 μg of retinol or 1 RAE as compared to calculation of RE values. The Institute of Medicine report[2] emphasizes that vitamin A intake from provitamin A carotenoids has been overestimated by previous assumptions made on carotenoid absorption from mixed meals including fruits and vegetables. Implications to the presentation of food composition databank information are significant in that reliable data on carotenoid composition of fruit and vegetables is still somewhat limited.

Dietary reference intake values (DRI) for vitamin A range from an adequate intake (AI) of 400 μg RAE d^{-1} for the 0- to 6-month infant to RDA values of 700 μg RAE d^{-1} for adult women and 900 μg RAE d^{-1} for adult men. The RDA increases to 1300 μg RAE d^{-1} for lactating women (19–50 years).[2] The tolerable upper intake level (UL) is 3000 μg RAE d^{-1} for adults (Table 1.2).

Functional roles of vitamin A and other retinoids continue to be identified as knowledge of their participation in cell growth and differentiation expands. Well-known functions of vitamin A include the visual cycle, effects on immune response, and gene-expression during embryonic development. Steps in the visual cycle include isomerization to 11-*cis*-retinol, transport by the interphotoreceptor-binding protein to the rod outer segment, enzymatic conversion to 11-*cis*-retinal, and formation of rhodopsin from opsin and 11-*cis*-retinal.[8] Specific sites of vitamin A action in immune response include action of T-helper and natural killer cells.

Identification of two families of nuclear receptors, retinoic acid receptors (RARs), and retinoid × receptors (RXRs) led to a clearer understanding of the role of vitamin A in cell differentiation. RARs bind with all-*trans*-or 9-*cis*-retinoic acid, and the RXRs bind only with 9-*cis*-retinoic acid, functioning in gene expression. Effects are highly varied and include the following:

1. Development of pancreatic a and b cells[18]
2. Up-regulation of insulin production[19]
3. Up-regulation of the genes for vitamin A transport proteins[20]
4. Downregulation of ornithine decarboxylase gene expression[21]
5. Increased insulin-stimulated glucose transport and GLUT 4 mRNA in L6 muscle cells[22]

With the expanding knowledge of the diverse functions of vitamin A, its role in child health and well-being becomes even more critical. Vitamin A deficiency continues to be the primary cause of blindness and chronic disease in young children in areas where dietary supply is insufficient. Fortification programs provide adequate vitamin A in supplementary form to large segments of the world's population. However, such programs need to be developed to counteract the significant public health impacts where nutritional deficiency still exists. The role of the food industry and public health organizations in this regard can still dramatically improve nutrition worldwide.

Table 1.2 Dietary Reference Intakes (DRIs) and Tolerable Upper Intake Levels (UL) for Vitamin A

Life stage	DRI (μg d^{-1})	UL
Infants (months)		
0–6	400	600
7–12	500	600
Children (years)		
1–3	**300**	600
4–8	**400**	900
Males (years)		
9–13	**600**	1700
14–18	**900**	2800
19–30	**900**	3000
31–50	**900**	3000
51–70	**900**	3000
>70	**900**	3000
Females (years)		
9–13	**600**	1700
14–18	**700**	2800
19–30	**700**	3000
31–50	**700**	3000
51–70	**700**	3000
>70	**700**	3000
Pregnancy (years)		
≤18	**750**	2800
19–30	**770**	3000
31–50	**770**	3000
Lactation (years)		
≤18	**1200**	2800
19–30	**1300**	3000
31–50	**1300**	3000

Bold type: Recommended dietary allowance; ordinary type: Adequate Intake (AI).[2]

Estimation of dietary intake or nonprovitamin A carotenoids has become more significant to public health as new functional roles are identified for carotenoids such as lutein and lycopene. Although β-carotene and several other carotenoids are clinically associated with lower risk of several chronic diseases, the RDI Panel on Antioxidants and Related Compounds[23] supported increased consumption of fruits and vegetables but did not set DRIs for β-carotene or other carotenoids. The panel did not recommend use of supplemental β-carotene other than as a source of provitamin A for the control of vitamin A deficiency. Excellent reviews are available that cover the expanding roles of vitamin A, other retinoids, and the carotenoids in prevention of disease and human development.[24–29]

Wide-spread vitamin A deficiency is still prevalent in many areas of the world. Since rice consumption is recognized as the most important source of human food, providing 80% of the caloric supply to over 50% of the world's population,[30,31] plant breeding and bioengineering efforts have centered on increasing the β-carotene content. Most notable is the genetic engineering advances leading to the development of Golden Rice recently reviewed by Lucca and colleagues[31] and Beyer and colleagues.[32] These reviews provide a current update on the efforts of many investigators to improve β-carotene availability by increasing the levels in the most consumed staple in the human's diet. Undoubtedly, Golden Rice will have major impact on vitamin A deficiency as its introduction into agricultural production proceeds.

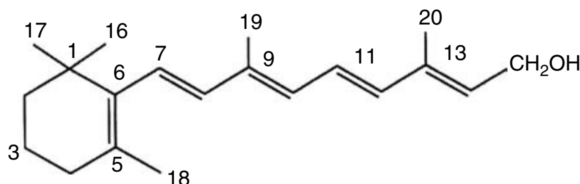


Figure 1.1 Structure of all-*trans*-retinol.

1.2 Properties

1.2.1 Chemistry

1.2.1.1 General properties

1.2.1.1.1 Vitamin A. The structure of all-*trans*-retinol (vitamin A) is given in Figure 1.1. Vitamin A refers to all isoprenoid compounds from animal products that possess the biological activity of all-*trans*-retinol. The retinol parent structure to most retinoids contains a substituted β-ionone ring (4-{2,6,6-trimethyl-2-cyclohexen-1-yl}-3-buten-2-one) with a side chain of three isoprenoid units linked at the 6-position of the β-ionone ring. The conjugated double-bond system includes the 5,6-β-ionone ring carbons and the isoprenoid side chain. Retinoids include all substances with vitamin A activity, some of which differ structurally from all-*trans*-retinol. Synthetic retinoids are increasingly being used for treatment of skin disorders such as acne and psoriasis. Effects of retinoids on cell differentiation will undoubtedly lead to many accepted therapeutic treatments that are now in the experimental stages. Such treatments include potential actions against various leukemias, malignancies, immunological abnormalities, and inflammatory conditions.

Important metabolites of all-*trans*-retinol are shown in Figure 1.2. Oxidation of the alcohol moiety of all-*trans*-retinol yields all-*trans*-retinal. Further oxidation produces all-*trans*-retinoic acid. In the visual cycle, all-*trans*-retinol is isomerized to 11-*cis*-retinol, which is either converted to a retinyl ester or transported to the outer rod segment and oxidized to 11-*cis*-retinal before combining with opsin to form rhodopsin.[8] Acetate and palmitate esters of all-*trans*-retinol are the primary commercial forms of vitamin A available to the pharmaceutical and food industry. Esterification greatly stabilizes the vitamin toward oxidation. The USP standard for vitamin A is retinyl acetate. This standard reference material is stable and supplied in ampules to allow quick turnover of the standard.

Physical properties of all-*trans*-retinol and closely related retinoids are provided in Table 1.3. Vitamin A is water insoluble and soluble in fats and oils, and most organic solvents.

1.2.1.1.2 Carotenoids. Over 600 carotenoids have been characterized. Nomenclature for the carotenoids specified by the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB) was reviewed by Weedon and Moss.[36] The reader is urged to read this source for an easy-to-follow description of the nomenclature rules. Organic synthesis of both the retinoids and natural carotenoids was covered in detail by Valla and colleagues.[37] Detailed information on the electrochemical properties of the carotenoids is available.[38]

The structures are formed by the head-to-tail linkages of eight isoprene units to provide a C_{40} skeleton. Lycopene (Figure 1.3) shows the acyclic hydrocarbon backbone chain. This compound is regarded as the prototype of the family.[36] Structural modifications of lycopene

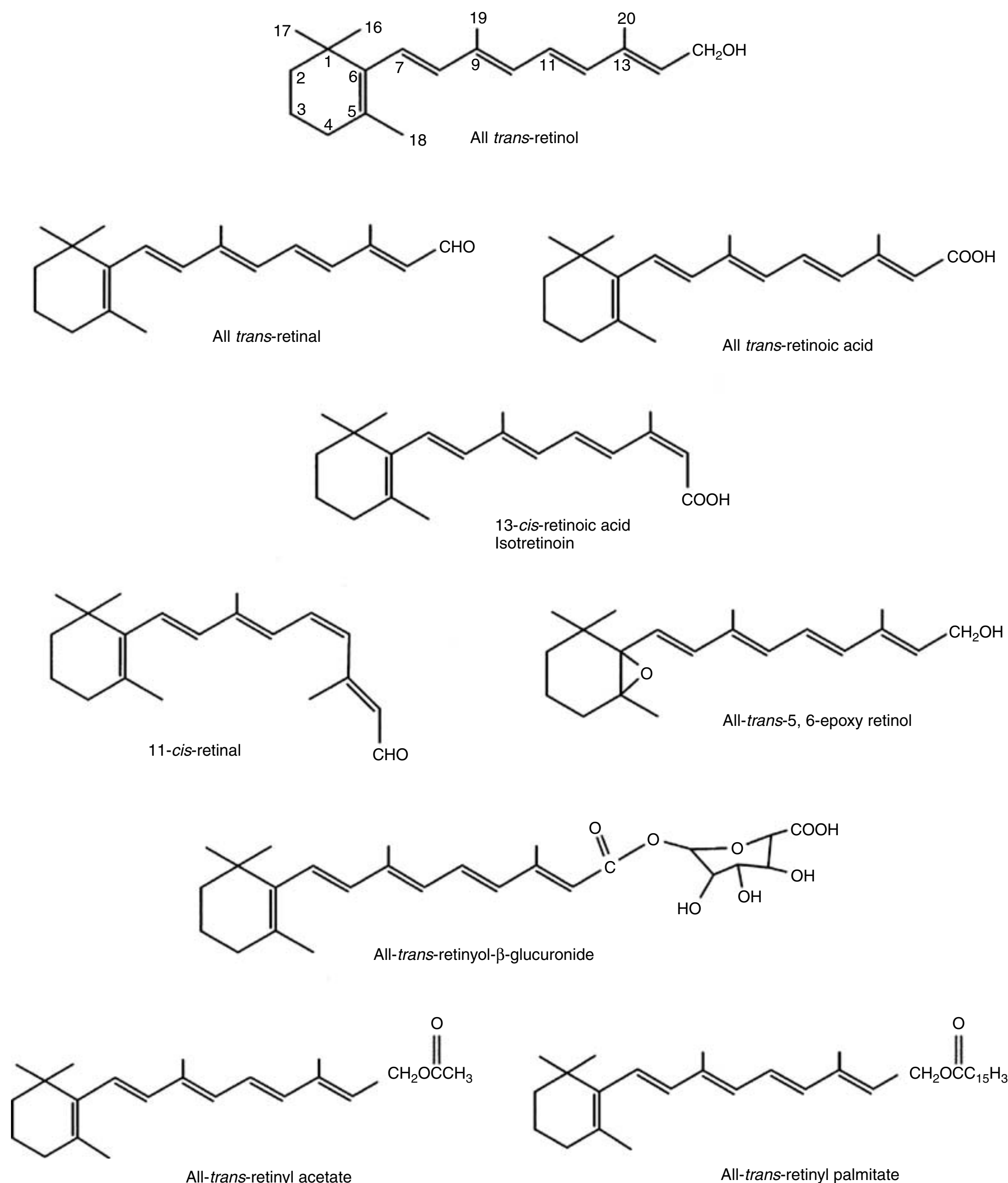


Figure 1.2 Structures of vitamin A metabolites and esters.

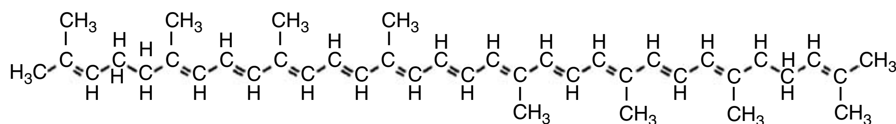
lead to the diverse nature of the carotenoids present in the plant kingdom. β-Carotene (Figure 1.3) is the most significant of the provitamin A carotenoids, characterized by the cyclicized β-ionone rings on both ends of the hydrocarbon chain. Structures of common carotenoids are shown in Figure 1.4. Addition of oxygenated functions to the molecule yields the xanthophylls. Oxygen functions include hydroxylation at the 3- or 4-position (lutein and β-cryptoxanthin) and ketolation (canthaxanthin) as well as formation of aldehydes, epoxy, carboxy, methoxy, and other oxygenated forms. Hydrocarbon carotenoids (lycopene, phytoene, and phytofluene), carotenal esters, and carotenol fatty acid esters are frequently found in plant materials.

Table 1.3 Physical Properties of Retinol and Other Retinoids

Substance[a]	Molar mass	Formula	Solubility	Melting point °C	Crystal form	UV absorption[b]			Fluorescence[c]	
						λ_{max} nm	$E_{1\,cm}^{1\%}$	$\varepsilon \times 10^{-3}$	Ex nm	Em nm
All-*trans*-retinol CAS No. 68-26-8 **10150**	286.46	$C_{20}H_{30}O$	Soluble in absolute alcohol, methanol, chloroform, ether, fats, oils	**62–64**	Yellow prisms	325 **325**	1845 **1810**	**[52.8]** **[51.8]**	**325**	470
13-*cis*-retinol	286.46	$C_{20}H_{30}O$		58–60		328	1689	[48.3]		
11-*cis*-retinol	286.46	$C_{20}H_{30}O$				319 **318**	1220 **1200**	[34.9] **[34.3]**		
All-*trans*-retinyl acetate CAS No. 127-47-9 **10150**	328.50	$C_{22}H_{32}O_2$		57–58	Pale-yellow prismatic	325 **325**	1560 **1590**	[51.2] **[52.2]**	325	470
All-*trans*-retinyl palmitate CAS No. 79-81-2 **10150**	524.88	$C_{36}H_{60}O_2$		28–29	Amorphous or crystalline	325	940	[49.3]	325	470
All-*trans*-retinal CAS No. 116-31-4 **8331**	284.44	$C_{20}H_{28}O$	Soluble in ethanol, chloroform, cyclohexane, petroleum ether, oils	61–64 (*trans*) 63.5–64.4 (11-*cis*)	Orange crystals	383 **368**	1510 **1690**	[42.9] **[48.0]**		
13-*cis*-retinal	284.44	$C_{20}H_{28}O$				375 **363**	1250 **1365**	[35.5] **[38.8]**		
11-*cis*-retinal	284.44	$C_{20}H_{28}O$		63.5–64.4	Orange prisms	380 **365**	878 **928**	[24.9] **[26.4]**		
All-*trans*-retinoic acid CAS No. 302-79-4 **8333**	300.44	$C_{20}H_{28}O_2$		180–182	Crystals	350	[1510]	45.3		
13-*cis*-retinoic acid CAS No. 4759-48-2 **8333**	300.44	$C_{20}H_{28}O_2$		174–175	Reddish-orange plates	354	[1325]	39.8		

[a] Common or generic name; CAS No: Chemical Abstract Service number, bold print designates the Merck Index monograph number.
[b] In ethanol; bold values in hexane; values in brackets are calculated from corresponding ε or $E_{1cm}^{1\%}$ values.
[c] In isopropanol.

Sources: Budavari, S., *The Merck Index*, 13th ed., Whitehouse Station, New Jersey, 2001; Barua, A. B., Olson, J. A., Furr, H. C., and van Breeman, R. B., In *Modern Chromatographic Analysis of Vitamins*, 3rd ed., De Leenheer, A. P., Lambert, W. E., and Van Bocxlaer, J. F., eds., Marcel Dekker, Inc., New York, 2000, chap. 1; Furr, H. C., Barua, A. B., and Olson, J. A., In *Modern Chromatographic Analysis of Vitamins*, 2nd ed., De Leenheer, A. P., Lambert, W. E., and Nelis, H. J., eds., Marcel Dekker, New York, 1992, chap. 1; Furr, H. C., Barua, A. B., and Olson, J. A., In *The Retinoids, Biology, Chemistry and Medicine*, Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds., Raven Press, New York, 1994, chap. 3; Olson, J. A., In *Handbook of Vitamins*, 3rd ed., Rucker, R. B., Suttie, J. W., McCormick, D. B., and Macklin, L. J., eds., Marcel Dekker, Inc., New York, 2001, chap. 1.



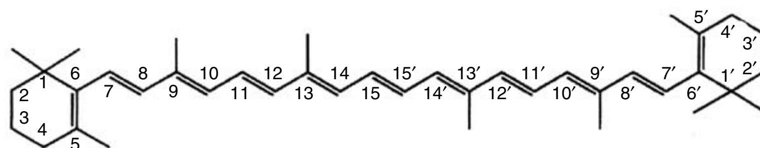


Figure 1.3 Structures of β-carotene and lycopene.

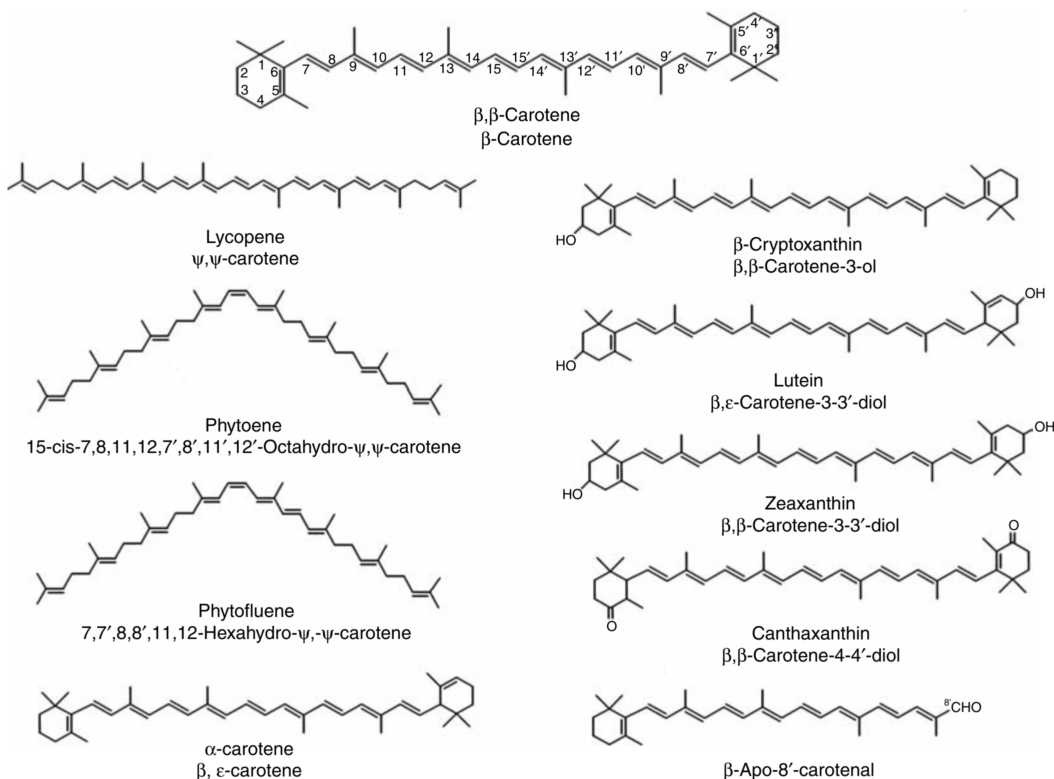


Figure 1.4 Structures of commonly occurring carotenoids.

Physical properties of several carotenoids are given in Table 1.4. β-Carotene and β-apo-8′-carotenal are important pigments added to margarine, salad dressings, and many other products to enhance color. When used in this respect, the provitamin A carotenoids can provide significant vitamin A activity to the food. In the poultry industry, xanthophyll concentrates derived from various natural products including the marigold are widely used as feed ingredients that enhance yellow to red pigmentation in poultry skin and egg yolks. Vitamin A activity of the products is not affected since the xanthophylls in such concentrates lack vitamin A activity.

Table 1.4 Physical Properties of Selected Carotenoids

Substance[a]	Molar mass	Formula	Solubility	Melting point (°C)	Crystal form	Absorbance λ_{max}	Absorbance $E^{1\%}_{1\,cm}$	$\varepsilon \times 10^{-3}$	Solvent
Provitamin A carotenoids									
β-Carotene	536.88	$C_{40}H_{56}$	Soluble in CS_2, benzene,	183	Red rhombic	425	—	—	Petroleum
CAS No. 7235-40-7			chloroform		square leaflets	453	2592	139	ether
1902						479	—	—	Cyclohexane
						452	2505	134	
α-Carotene	536.88	$C_{40}H_{56}$	Free soluble in CS_2,	187.5	Deep-purple	422	—	—	Petroleum
CAS No. 7488-99-5			chloroform; soluble		prisms	444	2800	150	ether
1901			in ether, benzene			474	—	—	
β-Cryptoxanthin	552.88	$C_{40}H_{56}O$	Freely soluble in	158–159	Red plates with	425	—	—	Petroleum
2676			chloroform, benzene,	(racemic)	metallic luster	452	2386	132	ether
			pyridine, CS_2	169 (natural)		479	—	—	Hexane
						450	2460	136	
γ-Carotene	536.88	$C_{40}H_{56}$	Somewhat less soluble	152–153.5	Red plates	437	2055	110	Petroleum
CAS No. 472-93-5			than β-carotene	(synthetic)	(synthetic)	462	3100	166	ether
1903				177.5 (natural)	Deep-red prisms (natural)	494	2720	146	
β-Apo-8′-carotenal	416.65	$C_{30}H_{40}O$	Freely soluble in	136–142	Powder with	457	2640	110	Petroleum
CAS No. 1107-26-2			chloroform; sparingly	(decomp)	dark metallic				ether
			soluble in acetone		sheen				
Other carotenoids									
Phytoene	5495					275	—	—	Petroleum
						285	1250	68.1	ether
						296	—	—	Hexane
						286	915	49.8	
Phytofluene	542.93	$C_{40}H_{68}$	Freely soluble in	B.P.	Pale-orange	331	—	—	Petroleum
CAS No. 540-05-6			petroleum ether,	140–185	viscous oil	348	1350	73.3	ether
7544			benzene; practically insoluble in H_2O, methanol, ethanol			367	—	—	

Lycopene CAS No. 502-65-8 **5650**	536.88	$C_{40}H_{56}$	Soluble in chloroform, benzene; insoluble in methanol, ethanol	172–173	Deep-red needles	444	—	—	Petroleum ether
						472	3450	185	
						502	—	—	
Lutein CAS No. 127-40-2 **10197**	568.88	$C_{40}H_{56}O_2$	Soluble in fats and fat solvents	190	Yellow prisms with metallic luster	421	—	—	Petroleum ether
						445	2550	145	
						475	—	—	
Zeaxanthin CAS No. 144-68-3 **10248**	568.88	$C_{40}H_{56}O_2$	Slightly soluble in petroleum ether, ether, methanol; soluble in CS_2, benzene, chloroform, pyridine, ethyl acetate	215.5	Yellow rhombic plates with steel-blue metallic luster	426	—	—	Petroleum ether
						452	2348	133	
						479	—	—	
						450	2540	144	
Canthaxanthin	564.85	$C_{40}H_{52}O_2$	Soluble in chloroform, oil; very slightly soluble in acetone	207–217 (decomp)	Violet	466	2200	124	Petroleum ether
Violaxanthin CAS No. 126-29-4	600.88	$C_{40}H_{56}O_4$	Soluble in alcohol, methanol, ether; almost insoluble in petroleum ether	200	Orange prisms	420	—	—	
						443	2550	153	Ethanol
						470	—	—	Hexane
						440	2550	153	
Neoxanthin	600.88					416	—	—	
						439	2243	135	Ethanol
						467	—	—	Hexane
						438	2470	148	
Astaxanthin CAS No. 472-61-7 **890**	596.85	$C_{40}H_{52}O_4$		182–183	Needles	472	2135	124	Petroleum ether

[a] Common or generic name; CAS No: Chemical Abstract Service number; bold print designates the Merck Index monograph number.

Sources: Bauernfeind, J. C., *Carotenoids as Colorants and Vitamin A Precursors, Technological and Nutritional Applications*, Academic Press, New York, 1981, Appendix; Budavari, S., The *Merck Index*, 13th ed., Merck and Company, Whitehouse Station, New Jersey, 2001; Britton, G., Liaaen-Jensen, S., and Pfander, H., *Carotenoids, Spectroscopy, Vol. 1B*, Birkhäuser Verlag, Boston, 1995, chap. 2; Committee on Food Chemicals Codex, *Food Chemicals Codex*, 5th ed., National Academy of Sciences, Washington, DC, 2004, p. 494; Friedrich, W., In *Vitamins*, Walter de Gruyter, Hawthorne, NY, 1988, chap. 2; Barua, A. B., Olson, J. A., Furr, H. C., and van Breeman, R. B., In *Modern Chromatographic Analysis of Vitamins*, 3rd ed., De Leenheer, A. P., Lambert, W. E., and Van Bocxlaer, J. F., eds., Marcel Dekker, Inc., New York, 2000, chap. 1.

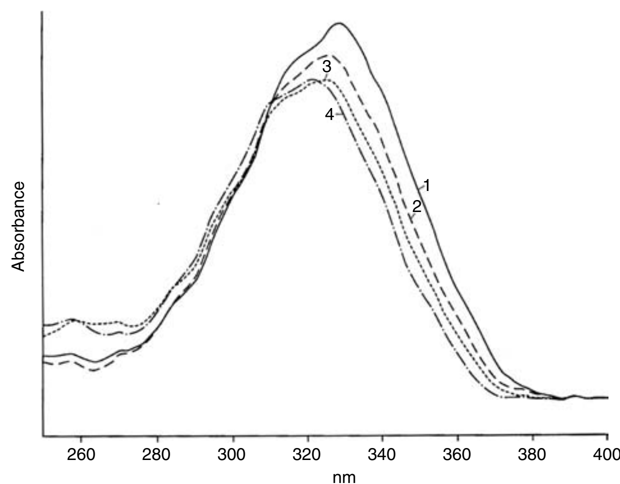


Figure 1.5 Spectra of retinol isomers. 1 = 13-*cis*-retinol, 2 = all-*trans*-retinol, 3 = 9,13-di-*cis*-retinol, 4 = 9-*cis*-retinol. (Reproduced with permission from Brinkmann, E., Dehne, L., Oei, H. B., Tiebach, R., and Baltes, W., *J. Chromatogr. A*, 693, 271, 1995.)

1.2.1.2 Spectral properties

Retinoids possess strong ultraviolet (UV) absorption properties owing to the conjugated double-bond system. Ultraviolet absorption maxima vary as structural variations are introduced to the parent all-*trans*-retinol. Structural variations in the large number of synthetic retinoids and effects on UV absorption were discussed in detail by De Leenheer and colleagues.[43] Maximal absorption occurs from 318 nm to greater than 360 nm (Table 1.3). Absorption maxima vary depending on the solvent and the presence of *cis*-isomers (*Z*–). Isomerization to the *cis*-forms lowers the absorption maxima and $E^{1\%}_{1\text{cm}}$ values relative to all-*trans*-retinol (Table 1.3).[34] The most common wavelength used for detection of all-*trans*-retinol after liquid chromatography (LC) resolution is 325 nm. The UV spectra of all-*trans*-retinol and its *cis*-isomers are provided in Figure 1.5.[44]

Fluorescence provides an advantageous alternative to UV absorption as a detection mode for specific retinoids. All-*trans*-retinol and retinyl esters possess excellent fluorescence properties at Ex λ from 325 to 330 nm and Em λ of 470 to 490 nm.[45] Fluorescence intensity is greater in nonpolar solvents, and normal phase systems with hexane-based mobile phases offer ideal detector response conditions for use of fluorescence compared to UV detection.[34] Most retinoids other than retinol and its esters do not fluoresce and oxidation of the alcohol results in loss of fluorescence; therefore, UV detection is used for quantitation of many synthetic retinoids and retinoic acid.[43]

The varied and bright pigmentation of the carotenoids results from the strong primary absorption in the visible region resulting from the long conjugated double-bond system. A characteristic UV absorption peak occurs in *cis*-isomers approximately 142 nm below the longest-wavelength absorption maxima of the all-*trans*-carotenoids.[46] The characteristic strong absorption between 400 and 500 nm is universally used for detection after LC resolution. Published absorbance parameters for common carotenoids are given in Table 1.4. The spectra commonly show two to three maxima in the visible range. The UV maximum in *cis*-isomers typically occurs between 330 and 340 nm with a downward wavelength shift of the entire spectra.[43,46] Characteristic spectra of β-carotene are shown in Figure 1.6.[47]

Absorption intensity is affected by the solvent or mobile phase composition carrying the carotenoid.[47,48] Published absorption maxima and the solvent and/or mobile phase are given in Tables 1.5[48] and 1.6.[49]

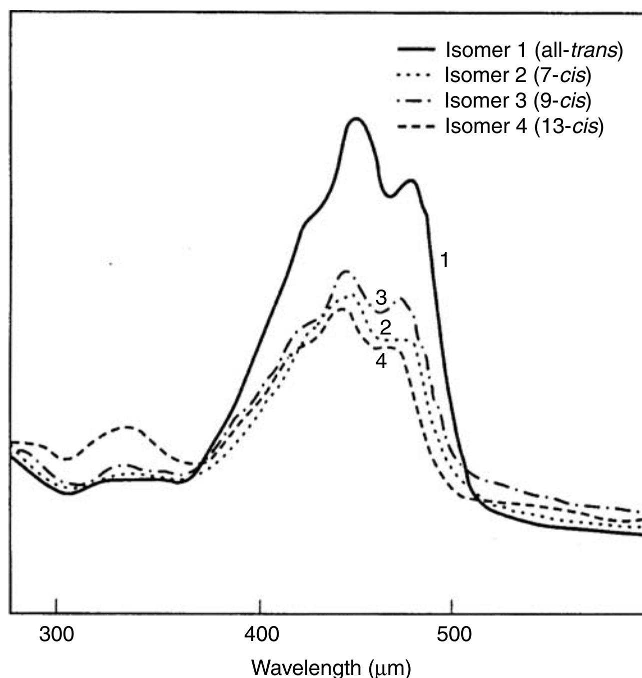


Figure 1.6 Spectra of β-carotene isomers. 1 = all-*trans*-β-carotene, 2 = 7-*cis*-β-carotene, 3 = 9-*cis*-β-carotene, 4 = 13-*cis*-β-carotene. (Reproduced with permission from Nyambaka, H. and Ryley, J., *Food Chem.*, 55, 63, 1996.)

Table 1.5 Comparison of λ Maxima for Carotenoids Showing Solvent Effects

	λ_{max} (nm)	
Carotenoid	MTBE in methanol[a]	Petroleum ether[b]
β-Carotene		
All-*trans*	450	453
9-*cis*	444	—
13-*cis*	443	—
α-Carotene		
All-*trans*	444	444
9-*cis*	441	—
13-*cis*	438	—
Zeaxanthin		
All-*trans*	450	450
9-*cis*	446	—
13-*cis*	443	—
Lutein		
All-*trans*	442	445
9-*cis*	438	—
13-*cis*	435	—
β-Cryptoxanthin		
All-*trans*	449	452
9-*cis*	444	—
13-*cis*	442	—

[a] Mobile phase consisted of 5–38% methyl-*tert*-butyl ether (MTBE) in methanol.[48]
[b] See Table 1.4.

Table 1.6 Absorption Maxima of α-Carotene Isomers in Pure Organic Solvents

Isomer	Absorption maxima[a]	
	Hexane	Petroleum ether
All-*trans*	267, nd, 421, (445), 474	267, 328, 420, (443), 472
13-*cis*	267, 331, 415, (438), 465	271, 329, 413, (436), 463
13′-*cis*	270, 330, 415, (437), 465	268, 330, 413, (435), 463
9-*cis*	258, 330, 417, (440), 469	255, 328, 416, (439), 467
9′-*cis*	258, 328, 418, (441), 469	258, 328, 415, (439), 467
	MTBE	Ethanol
All-*trans*	268, nd, nd, (445), 474	267, nd, nd, (447), 474
13-*cis*	270, 331, nd, (438), 466	267, 331, nd, (439), 466
13′-*cis*	269, 331, nd, (438), 465	269, 331, nd, (438), 465
9-*cis*	259, 329, 420, (442), 470	258, 331, 420, (442), 470
9′-*cis*	266, 329, 420, (442), 470	259, 328, nd, (442), 470
	Dichloromethane	Acetone[b]
All-*trans*	271, nd, 432, (456), 484	330, nd, (448), 476
13-*cis*	273, 337, 428, (449), 476	332, nd, (440), 468
13′-*cis*	272, 337, 424, (448), 475	332, nd, (440), 467
9-*cis*	261, 339, 428, (450), 479	332, nd, (443), 471
9′-*cis*	262, 336, 429, (452), 480	332, nd, (444), 472

nd: No maximum was detected by the spectrophotometer.

[a] Units are nm. Values in parentheses are the primary l max. The nd designation indicates that no maximum was detected.

[b] Absorbance at ≤330 nm interfered with the detection of the overtone (~265 mn).

Source: Emenhiser, C., Englert, G., Sander, L. C., Ludwig, B., and Schwartz, S. J., *J. Chromatogr. A*, 719, 333, 1996.

1.2.2 *Stability*

Several excellent reviews exist on the stability of all-*trans*-retinol and the carotenoids. Particularly useful reviews to the analyst include those by Ball,[45] Schiedt and Liaaen-Jensen,[50] and Delgado-Vargas and colleagues.[51] Similar chemical properties of the vitamin A retinoids and provitamin A carotenoids contribute to common problems encountered during analytical determination. These include instability when isolated from the biological matrix, susceptibility to isomerization to *cis*-isomers with lower biological activity, and different absorption properties, and susceptibility to oxidation. Owing to the general lack of stability, artifacts formed during sample storage, extraction, and analysis can make analytical results difficult to interpret and even meaningless.

Schiedt and Liaaen-Jensen[50] provided the following general precautions necessary to minimize carotenoid changes throughout the analysis:

1. Exclude oxygen; air should be replaced by vacuum or inert gas.
2. Addition of antioxidants such as butylated hydroxytoluene (BHT), pyrogallol, or ascorbyl palmitate is necessary before saponification. Low levels of antioxidants in extracting solutions and mobile phases are often added to protect the retinoids and carotenoids from oxidation.
3. *Trans-* to *cis*-isomerization (E/Z) is promoted at higher temperatures. Therefore, use of the lowest practical temperature is recommended, and use of solvents with low boiling points is preferred. For rotary evaporation, 40°C should not be exceeded. Solutions should be stored at –20°C and preferably at lower temperature.
4. All sunlight should be avoided. Analytical steps should be completed in dim light, diffused sunlight, or under gold fluorescent light. Solutions should be stored in low

actinic glassware whenever possible. Isomerization rapidly occurs through light activation and is a common source of *cis*-isomer formation in biological sample extracts.

5. Acid must be avoided. All solvents must be acid-free. Addition of triethylamine (TEA) at 0.001% is useful to neutralize low acid levels occurring in some solvents.
6. Alkaline conditions can lead to base-catalyzed isomerization.

E/*Z* isomerization is difficult to completely avoid during sample handling and determinative analysis. In addition, 5,6-epoxides readily rearrange to the 5,8-epoxide in the presence of acids.[50] Conversion of all-*trans*-retinol to *cis*-isomers is higher in chlorinated solvents as compared to nonchlorinated solvents.[52] Iron and copper supplementation of foods can increase the rate of isomerization.[52] Studies by Landers and Olson[53] showed that retinol and retinyl palmitate were isomerized much less extensively in hexane solution compared to chloroform or methylene chloride when exposed to white light. Isomers included 13-*cis*, 11-*cis*, 9-*cis*, and 7-*cis* in order of decreasing amounts. Gold light exposure did not produce isomerization. Gold lighting by the Sylvania F40/GO gold fluorescent light or equivalent is considered essential for laboratories routinely involved in vitamin A or carotenoid assay.

Stability in Plasma: Peng and colleagues[54] studied the stability of all-*trans*-retinol in plasma. Their data showed that blood could be stored on ice in the dark for at least 24 h before separation of the plasma. Plasma could be stored up to 1 year at 220°C or lower without loss of vitamin A activity. Scita[55] showed β-carotene to be fairly stable over 24 h when micellar solutions in aqueous medium were held at 37°C in the dark in a 5% CO_2 and air atmosphere. Exposure to UV light and fluorescent light was highly degradative. The addition of BHT or tocopherol at 1 mM concentrations significantly reduced the loss of β-carotene. Tocopherol was approximately three times as effective as BHT in slowing oxidation. BHT (0.025%) did not prevent slow β-carotene loss under storage at 220°C in the dark under nitrogen. Rates of loss were 1.5% per month and 1.1% per month in the absence or presence of the antioxidant. Su and colleagues[56] followed the stability of various antioxidants including retinol, several carotenoids and tocopherols in human plasma under laboratory fluorescent lighting at ambient temperature. No significant changes occurred within 72 h ($p < .05$) for all-*trans*-retinol, β-carotene, *cis*-lycopene, or lutein/zeaxanthin. After extraction from the plasma, the analytes were quite stable at ambient 4°C and –20°C; however, variability at room temperature storage was greater than in samples, stored frozen or refrigerated. Thus, the authors recommended that extracts be stored cold. Craft and colleagues[57] reported similar observations for carotenoids in extracted plasma. Ihara and colleagues[58] reported excellent stability for retinol in lyoplilized vitamin-enriched serum that was stored at –20°C for 12 months.

Stability in Foods: Many studies exist in the literature on the stability of all-*trans*-retinol, retinyl palmitate, and β-carotene in foods during processing and storage. Conversely, little information exists on the stability of other carotenoids such as lycopene. This is largely the result of difficulties that still exist in most laboratories to accurately assess carotenoid profiles and changes in all-*trans*-isomers to multiple *cis*-isomeric forms. Shi and LeMaguer[59] reviewed changes in lycopene in tomatoes caused by food processing. They summarized lycopene stability as follows:

1. Primary degradation routes for all-*trans*-lycopene in tomatoes during processing are isomerization and oxidation.
2. Heat induces isomerizations.
3. The amount of *cis*-isomers increases with temperature and processing time.

4. Dehydrated and powdered tomatoes have poor lycopene stability.
5. Frozen and heat-sterilized foods have excellent lycopene stability.

One of the best reviews on all-*trans*-retinol and β-carotene stability in foods was presented by Ball,[45] and the reader is urged to access this reference for specific literature references. Of significance, the following observations are evident in the published studies on the stability of all-*trans*-retinol, provitamin A active carotenoids, and other carotenoids in food:

6. Conversion of all-*trans*-isomers to *cis*-isomers occurs easily. Light, acids, metals, lipoxygenase, and heat processing, acting independently or sometimes synergistically, can produce rapid isomerization.
7. All-*trans*-retinol and the carotenoids are oxidatively unstable. The presence of autoxidizing lipid systems can lead to rapid loss of vitamin A activity. Some carotenoids are active singlet oxygen quenchers. This ability is directly related to the number of double bonds in the conjugated double-bond system. β-carotene, lutein, and other carotenoids preferentially react with singlet oxygen with conversion to triplet state forms.[60] The excited triplet state carotenoid thermally disperses the excess excited state energy through a chemical reaction that destroys the carotenoid.[61] Degradation products include various cleavage products.
8. In addition to singlet oxygen quenching, carotenoids scavenge free radicals at low oxygen pressures of less than 155 mmHg and act as primary chain-breaking antioxidants. In this capacity, the primary mode of β-carotene is through trapping of peroxyl radicals. Mechanisms include addition of the radical at the 5,6-double bond of the carotenoid with formation of epoxides.[61]
9. All-*trans*-retinol and the carotenoids become more unstable as the food matrix is disrupted or the compounds are removed from the matrix by extractions before analytic quantitation. Destruction of the sample matrix can liberate lipoxygenases that catalyze isomerization.
10. Blanching of plant products before freezing inactivates lipoxygenase and removes oxygen from the tissue. Thus, carotenoids are protected from oxidative degradation during freezer storage.
11. Air drying or freeze drying can lead to large losses in all-*trans*-retinol or the carotenoids. Storage of freeze-dried material, because of the open, porous nature of the product, requires removal of oxygen with inert gas flushing or vacuum to stabilize the vitamin A activity. Losses can be considerable during the drying process. The analyst should avoid dehydration of the sample, if possible. Low-temperature freezing of samples in evacuated, light protected containers is preferable.
12. All-*trans*-retinol and the carotenoids are relatively stable at alkaline pH. Therefore, saponification can be used for sample extraction if the saponification vessel is evacuated and protected from light. Ambient temperature saponification is often used to slow isomerization reactions that are more predominant at elevated temperatures.
13. All-*trans*-retinyl palmitate and all-*trans*-retinyl acetate are the common commercial forms of vitamin A used for food fortification and by the pharmaceutical industry. The ester forms are more stable to oxidation. However, isomerization can readily occur. Most LC procedures will resolve the all-*trans* from the *cis*-isomers, which is essential to accurately assess biological activity. Encapsulation of commercial preparations inhibits oxidation and isomerization. Antioxidants including tocopherols and other free-radical interceptors are usually added to vitamin A concentrates to inhibit oxidation.

Because of the significance of vitamin A fortification of dairy products and its addition to infant formula, many studies have been completed to determine the stability under a variety of processing and storage conditions for these products. Some of these studies are summarized in Table 1.7.[62–81] Likewise, carotenoid stability has been defined in many fruits and vegetables under various storage and processing conditions. Selected references dealing with carotenoid stability are summarized in Table 1.8.[82–100]

Table 1.7 Stability Studies on Vitamin A in Foods and Pharmaceuticals

Matrix and analyte	Process	Results	References
Dairy products			
Milk powders, RA, RP[a]	Storage at ambient temperature	Retinyl esters degraded 25%–47%. Most loss occurred during the first 6 months of storage	*New Zeal. J. Dairy Sci. Technol.*, 18, 21, 1983[62]
UHT milk, RA, RP	Storage at ambient and 35°C	Retinyl esters degraded over 28 weeks. At ambient temperature, loss was 35%–47%. Greater loss was at 35°C. Most loss occurred within 8 weeks storage	*J. Micronutr. Anal.*, 1, 13, 1985[63]
Low fat and skim milk, RP	Storage under fluorescent light at 4°C	Light photoxidation occurred under fluorescent lighting at a faster rate in skim compared to 2% milk. High PUFA carrier oils increased the rate of photoxidation compared to more saturated oils	*J. Dairy Sci.*, 69, 2038, 1986[64]
Skim milk, RP	Storage under fluorescent light at 4°C	All-*trans*-RP and 13-*cis*-RP decreased; whereas, 9-*cis*-RP increased over 48 h. Approximately 40% loss of RP was noted.	*J. Food Sci.*, 51, 1456, 1986[65]
Low fat milk, RP	Influence of milk fat, solids and light intensity on stability at 4°C	RP loss was less in whole milk compared to skim; addition of 1% NFMS did not protect RP; increasing light intensity increased rate of loss	*J. Dairy Sci.*, 69, 2779, 1986[66]
UHT milk, RP	Effect of fat level on UHT milk stored in the dark at 26°C for 3 weeks	Less loss was noted in fat >3%; retention stabilized after 2 weeks.	*J. Dairy Sci.*, 69, 2052, 1986[67]
UHT milk, RP	Stability in fortified and unfortified UHT milk stored at 23°C for 15 weeks	Losses in fortified UHT were >70%, less loss occurred for natural RP, but results were not reproducible	*J. Dairy Sci.*, 69, 2045, 1986[68]
Skim milk, RP	Effect of the vitamin carrier was studied during storage at 4°C and 35°C over 48 h under fluorescent light	Greater loss occurred with corn oil compared to coconut oil. Degradation rate decreased with homogenization pressure. Isomerization was more rapid at 35°C compared to 4°C	*J. Dairy Sci.*, 70, 13, 1987[69]
Skim milk, RP	Storage under retail fluorescent lighting in glass, polyethylene and paperboard containers at 7–10°C over 4 days	The rate of loss of all-*trans*-RP was first order in glass and polyethylene, no loss was observed in paperboard. 9-*cis*-RP was formed in glass and polyethylene containers	*J. Agric. Food Chem.*, 36, 592, 1988[70]

Continued

Table 1.7 (Continued)

Matrix and analyte	Process	Results	References
Dairy products (*Continued*)			
2% and skim milk, RP	Photoxidative stability under fluorescent lighting of milk in 1 gal polyethylene containers at 5°C for 72 h	A 69% loss occurred compared to 15% loss in a nonilluminated control	*J. Food Prot.*, 54, 113, 1991[71]
1% milk, retinol, RP	Stability of RP and indigenous retinol was followed in milk containing various emulsifiers	Added RP was as stable as retinol when butter oil was used as the emulsifier. The type of emulsifier did not affect stability; however, RP was more stable in most systems than indigenous retinol	*J. Dairy Sci.*, 73, 1485, 1990[72]
UHT whole milk, retinol	Retinol stability was followed under 30°C, 40°C and 50°C for 3 months and under frozen storage	Loss of up to 59% incurred at elevated storage temperatures, 17%–18% less occurred after 8 months storage at –20°C	*Z. Lebensm. Unters. Forsch.*, 195, 562, 1992[73]
Skim milk, RP	Stability studies on the effect of AA on RP stability under fluorescent light	AA inhibited light-induced isomerization of all-*trans*-RP	*J. Food Sci.*, 63, 597, 1998[74]
Milk, processed products, retinol	Effects of HTST, UHT, and sterilization on all-*trans*- and 13-*cis*-retinol	13-*cis*-retinol increase in the following order 72°C. 15 s < 72–76°C, 15 s < UHT. Fermented products contained variable levels of 13-*cis*-retinol	*J. Diary Res.*, 65, 253, 1998[75]
Infant formula, RP	RP stability during 12 months storage at 20°C, 30°C, and 37°C	RP losses were variable from manufacturer to manufacturer. Losses increased as storage temperature increased. Most products maintained minimum recommended levels to overages added	*J. Dairy Res.*, 67, 225, 2000;[76] *J. Food Sci.*, 65, 1052, 2000[77]
Other products			
Corn flakes, RP	RP stability during storage at ambient and 45°C	After 6–8 weeks, 90% of RP was lost except in cereals fortified with a vitamin fortification complex. Even at ambient temperature, the large surface area of corn flakes promotes RP degradation	*J. Food Sci.*, 65, 1216, 2000[78]
Model system, retinol, RP	Effect of UVA and UVB radiation on retinol and RP in sunscreen formulations	Butyl methoxydibenzolymethane showed the strongest protective effect retinol and RP. Butylated hydroxy toluene inhibited photodegradation of both vitamin forms dissolved in octyl octanoate.	*Int. J. Pharm.*, 240, 85, 2002[79]
Model system, retinol	Effect of cyclodextrins on retinol stability in aqueous ophthalmic solutions	Formation of inclusion complexes with cyclodextrins stabilized and solubilized all-*trans*-retinol in aqueous solutions	*J. Inclus. Phenom. Microcyclic Chem.*, 44, 155, 2002[80]
Creams and solutions, RP, retinoic acid	Storage under light and dark at 2°–8°C for 90 days	Rapid destruction under sunlight storage was observed. Protection from light resulted in >86% retention of all-*trans*-retinoic acid and >73% retention of RP in all formulations	*J. Pharmaceut. Biochem. Anal.*, 23, 147, 2000[81]

[a] RP: retinyl palmitate; RA: retinyl acetate.

Table 1.8 Stability Studies on Carotenoids in Foods

Matrix and analyte	Process	Results	References
Vegetables, total carotene	Solar drying	Total carotene was most stable under dehydration by an enclosed solar drier with shade. Direct sun exposure decreased retention. Retention in various vegetables ranged from 4.8% to 27%	*J. Food Sci.*, 46, 1288, 1981[82]
Sweet potatoes, *trans*-β-carotene	Sweet potato processing	Percentage loss was 8 for steam injection, 19.7 for canning, 20.5 for dehydration, 22.7 for microwaving and 31.4 for baking. Heat-processing formation of 13-*cis*-β-carotene	*J. Agric. Food Chem.*, 36, 129, 1988[83]
Carrots, broccoli, spinach, total carotene	Effects of freezing, thawing, cooking, dehydration	Frozen and thawed vegetables exposed to home preparation conditions retained carotene levels for up to 4 h. Dehydration significantly reduced total carotene content. Cooking did not decrease carotene content	*J. Food Sci.*, 52, 1022, 1987[84]
Tomatoes, green vegetables, epoxy carotenoids, and hydrocarbon carotenoids	Food preparation effects on tomatoes, broccoli, green beans, and spinach	Epoxy carotenoids (lycopene 1,2-epoxide, lycopene 5,6-epoxide, lutein epoxide, etc.) were more sensitive to heat processing than lutein and hydrocarbon carotenoids including α- and β-carotene and lycopene	*J. Agric. Food Chem.*, 40, 390, 1992[85]
Brazilian vegetables, *cis*-isomers of β-carotene	Occurrence of *cis*-β-carotene isomers, natural and formation during cooking	13-*cis*-β-carotene was found in all cooked vegetables. Without resolution and quantitation of the 13-*cis*-β-carotene, vitamin A activity is overestimated	*J. Agric. Food Chem.*, 46, 3081, 1998[86]
Carrot pulp, α- and β-carotene, lutein	Processing of carrot pulp and storage stability	Spray dried carrot pulp stored at temperatures up to 45°C for 12 weeks showed that all-*trans*-β-carotene degrade to *cis*-isomers to a greater extent than all-*trans*-α-carotene and all *trans*-lutein. 13-*cis*-α-carotene and 13-*cis*-β-carotene were predominant isomers found in carrot pulp stored in the dark. Light storage caused the 9-*cis*-isomers to predominate	*J. Agric. Food Chem.*, 46, 2312, 1998[87]
Carrots, α-and β-carotene	Food service cooking	Greatest stability of α- and β-carotene occurred with water-cooking without pressure. Various preparation methods led to losses of 13.6%–34.3% of total (α- + β-) vitamin A activity	*Food Chem.*, 61, 145, 1998[88]
Canola oil, β-carotene	Storage of fortified canola oil	Storage of canola oil under light at 30°C produced 13%–16% loss of added β-carotene. Losses were similar in high oleic canola oil	*J. Food Sci.*, 64, 996, 1999[89]
Broccoli, carrots, green beans, all-*trans*-β-carotene	Processing and storage studies	Storage of the fresh product in Ziploc® bags at 4°C maintained β-carotene for 3 weeks (broccoli, green beans) and 6 months for carrots. Fresh vegetables reatined >70% of β-carotene when blanched by steam and quick frozen. Microwave cooking had no effect	*J. Food Sci.*, 64, 929, 1999[90]

Continued

Table 1.8 (Continued)

Matrix and analyte	Process	Results	References
Tomato, lycopene, lutein, β-carotene	Heat processing effects on carotenoids	Commercial processing included washing, chopping, hot break extraction, sieving, vacuum evaporation, filling, sterilization at 100°C for 30 min. No change was noted in lycopene or lutein, all-*trans*-β-carotene decreased by approximately 60%	*J. Agric. Food Chem.*, 48, 2075, 2000[91]
Carrot chips, α- and β-carotene, *cis*-9-β-carotene	Deep-fried carrot chip processing	Dehydration (before frying) did not decrease vitamin A activity when presoaked in sodium metabisulfite.	*J. Agric. Food Chem.*, 49, 3253, 2001[92]
Model system, lycopene	Effect of illumination	Lycopene degradation at 25°C in a thin-film under illumination was first order. 5-*cis*-, 9-*cis*-, 13-*cis*- and 15-*cis*-isomers were present at variable concentrations over 144 h of illumination	*Food Chem.*, 78, 425, 2002[93]
Red pepper, β-carotene	Effect of blanching, frozen storage, AA addition on stability	Increasing AA concentration protected β-carotene during storage of paprika powder. Blanching at 70°C for 15 s or 30 s decreased β-carotene by 15 and 22%, respectively	*J. Nutr. Biochem.*, 3, 124, 1992[55]; *Nahrung*, 46, 308, 2002[94]; *Food Sci. Tech. Int.*, 8, 55, 2002[95]
Carrot juice, β-carotene, 13-*cis*- and 9-*cis*-β-carotene	Effect of blanching, pasteurization and sterilization or *cis*-isomer formation	Blanching of carrots affected subsequent levels of *cis*-isomers in pasteurized and sterilized juices. 9-*cis*-β-carotene was found only in sterilized juice of blanched carrots. Levels of *cis*-13-β-carotene reached 4.9–5.6% of total β-carotene in heat-processed juices	*Food Chem.*, 83, 609, 2003[96]
Orange juice, β-carotene, β-cryptoxanthin, zeaxanthin, lutein	Refrigerated storage of high pressure treated orange juice	Storage for 10 days at 4°C resulted in the decreases in vitamin A activity. Up to 43% of total vitamin A activity loss was attributed to changes in β-carotene concentration.	*J. Agric. Food Chem.*, 51, 647, 2003[97]
Tomato juice, β-carotene, lycopene, lutein, *cis*-isomers	Stability during storage under light and dark at 4°–35°C for 12 weeks	All-*trans*-lutein was not detected after storage at 4°C and 25°C after 5 weeks or 37°C after 4 weeks. *cis*-isomers of lutein also degraded rapidly. Formation of 9-*cis*- and 13-*cis*-β-carotene occurred through 10 weeks of storage and then concentrations decreased. All-*trans*-lycopene followed similar decreasing trends as β-carotene and lutein. 13-*cis*-lycopene was more stable than 5-*cis*-, 9-*cis*-, 15-*cis*-, and di-*cis*-lycopene.	*Food Chem.*, 90, 837, 2005[98]
β-carotene nanodispensions	Preparation of β-carotene nanodispensions and stability at 4°C	Percentage retention of β-carotene in nanodispensed form prepared by emulsification-evaporation ranged from 25% to 56% after storage at 4°C in amber bottles	*Food Chem.*, 92, 661, 2005[99]
Asian vegetables, β-carotene, 9-*cis*-, 13-*cis*-, 15-*cis*-β-carotene	Stability during frying	The following retentions were found after frying at 160°C for 1 min: sweet potato (81–95%), tomato (73–95%), bell pepper (83–98%). 13-*cis*-β-carotene was the predominant *cis*-isomer detected after frying	*J. Food Compos. Anal.*, 19, 562, 2006[100]

1.2.3 Bioavailability

Establishment of the μg retinol activity equivalent (μg RAE) (Section 1.1) by the Institute of Medicine[2] takes into account the current understanding of bioavailability of the carotenoids with consideration of their conversion to all-*trans*-retinol and absorption. Studies since publication of the 1989 National Research Council Recommended Dietary Allowances[101] that utilized RE, led to the adopted changes.

Provitamin A–active carotenoids include β-carotene, α-carotene, γ-carotene, and β-cryptoxanthin. *Cis*-isomers are less active than the all-*trans* naturally occurring carotenoids. After absorption, biological activity depends on the presence of one, nonhydroxylated β-ionone ring in the provitamin A carotenoid structure. Thus, acyclic hydrocarbon carotenoids such as lycopene do not possess vitamin A activity. Compounds that have one hydroxylated ring (β-cryptoxanthin) have approximately 50% of the biological activity of β-carotene. Hydroxylation of both β-ionone rings (lutein) leads to complete inactivity from a vitamin A functional standpoint. Conversion of provitamin A carotenoids to retinol is believed to occur through central and eccentric cleavage. Oxidative central cleavage at the 15,15′ double bond yields two moles of retinol per mole of β-carotene. Eccentric or random cleavage provides only one to two moles of retinol per mole of β-carotene, depending on the cleavage sites.[101] Bioavailability is influenced greatly by absorption properties of the carotenoids, vitamin A status, metabolic digestive disorders, the food matrix, and level of fat in the meal. Certain dietary components including fiber may interfere with absorption.[102–104]

Borel[104] reviewed factors that impact the intestinal absorption of high lipolytic food microconstituents such as the carotenoids. Such factors include transfer of the carotenoids into the fat phase of the meal in the stomach, distribution of the carotenoids in dietary lipid droplets (surface for polar carotenoids, core for apolar carotenoids), their ability to transfer to micelles in the duodenum and absorption (most likely passive). *Cis*-isomers are less readily absorbed than *trans*-isomers. Cooking usually improves bioavailability owing to more efficient extraction from the food matrix during digestion. Fat in the digesta also improves extractability and, thus, absorption.

1.3 Methods

Methodology for analysis of vitamin A and the carotenoids includes classical colorimetric, spectrophotometric, fluorometric, paper, and open-column and thin-layer chromatographic methods. With the rapid development of LC methodology over the past 30 years, most analysts now rely on LC, sometimes coupled with mass spectrometry, for assay and/or characterization of retinoids and carotenoids. Development of engineered reversed-phase supports by the National Institute of Technology (NIST)[105] resulted in the availability of a C_{30} support with greatly improved chromatographic parameters compared to previously available reversed-phase supports for carotenoid resolution. Use of the C_{30} support has been extensively incorporated into carotenoid and other fat-soluble vitamin studies ever since it became commercially available. Characteristics of the C_{30} support are discussed more fully in Section 1.3.2.2.2. Because of the large number of investigators involved in retinoid and carotenoid research, many excellent reviews of methods exist. Selected reviews on retinoid assay were written by Wyss,[106,107] Bhat and Sundaresan,[108] De Leenheer et al.[43] and Furr et al.[109] Methodology reviews on the retinoids and carotenoids include Parrish et al.,[110] Thompson,[111] Ball,[45] Tee and Lim,[112] Eitenmiller and Landen,[113] Barua et al.[34] and, Delgado-Vargas.[51] Those specific for carotenoid analysis include Rodriguez-Amaya,[114,115] Craft,[116] De Leenheer et al.,[117] Tsukida,[118] Khachik et al.,[119] O'Neil and Schartz,[120] Su et al.,[121] and Rodriguez-Bernaldo de Quirós and Costa.[122] Sander et al.,[123] reviewed applications of the C_{30} stationary phase for analysis of fat-soluble vitamins.

Table 1.9 summarizes various regulatory and handbook methods. Methods specific for retinol or its esters are often based on spectrophotometry. Erroneous results are common unless material under analysis is a relatively concentrated source of all-*trans*-retinol or its esters. Carotenoids from fruits and vegetables or from mixed foods should be assayed by LC. Other methods for food analysis can overestimate vitamin A activity because biologically inactive carotenoids are difficult to completely remove from the provitamin A carotenoids by open-column chromatography employed in the colorimetric or spectrophotometric methods.

Table 1.9 Regulatory and Handbook Methods for Analysis of Vitamin A and Carotenoids

Source	Form	Methods and application	Approach	Most current cross-reference
***U.S. Pharmacopeia*, National Formulary, 2006, USP 29/NF 24 Dietary Supplements Official Monographs**[124]				
1. Pages 2387–2388, 2389	Retinol Retinyl acetate Retinyl palmitate β-carotene	Vitamin A in oil-soluble vitamin capsules/tablets	LC, retinol 325 nm Spectrophotometric β-carotene 452 nm	None
2. Pages 2390–2391, 2394–2397, 2407–2409	Retinol Retinyl acetate Retinyl palmitate β-carotene	Vitamin A in oil- and water-soluble vitamin capsules/tablets, oral solution w/wo mineral	LC, retinol 325 nm Spectrophotometric β-carotene 452 nm	None
3. Pages 262–263	β-carotene	β-carotene capsules	Spectrophotometric 452 nm	None
4. Page 2258	Retinol Retinyl acetate Retinyl palmitate	Vitamin A (NLT 95.0% of label claim)	Spectrophotometric 325 nm	None
5. Page 2258	Retinol Retinyl acetate Retinyl palmitate	Vitamin A capsules	Spectrophotometric 325 nm	None
British Pharmacopoeia, 2007[125]				
1. Pages 2156 and 2157	Natural retinyl esters	Vitamin A ester (natural)	Spectrophotometric 326 nm	None
2. Pages 2157–2159	Retinyl acetate Retinyl palmitate Retinyl propionate	Natural vitamin A concentrate (oily form)	Spectrophotometric 325 nm LC 325 nm	None
3. Pages 1797–1798	Retinyl acetate Retinyl palmitate Retinyl propionate	Synthetic vitamin A concentrate (oily form)	Spectrophotometric 326 nm LC 325 nm	None
4. Pages 1798–1799	Retinyl acetate Retinyl palmitate Retinyl propionate	Synthetic vitamin A concentrate (powder form)	LC 325 nm	None
5. Pages 1799–1800	Retinyl esters	Synthetic retinol concentrate (solubilisate/emulsion)	Thin layer or LC 325 nm	None
6. Pages 2987–2989	Retinyl acetate Retinyl Palmitate Retinyl propionate	Paediatric vitamins A, C, and D oral drops	Spectrophotometric 325 nm LC 325 nm	None

Table 1.9 (Continued)

Source	Form	Methods and application	Approach	Most current cross-reference
***AOAC Official Methods of Analysis*, 18th ed., 2005**[126]				
1. 45.1.01	Retinol	AOAC Official Method 960.45 Vitamin A in margarine (surplus 1980)	Spectrophotometric	
2. 45.1.01.A	Retinyl palmitate	AOAC Official Method 2002.06 Retinyl palmitate in fortified fluid milk	LC 325 nm	*J. AOAC Int.*, 86, 375, 2003[160]
3. 50.1.02	Retinol	AOAC Official Method 992.04 Vitamin A in milk-based infant formula	LC 340 nm	*J. AOAC Int.*, 76, 399, 1993[127]
4. 50.1.03	Retinol	AOAC Official Method 992.06 Vitamin A in milk-based infant formula	LC 325 nm	
5. 45.1.02	Retinol β-Carotene	AOAC Official Method 974.29 Vitamin A in mixed feed, premixes, and feeds Carr-Price	Colorimetric 620 nm—Retinol 440 nm—β-carotene	
6. 45.1.03	β-Carotene	AOAC Official Method 941.15 Carotene in fresh plant materials and silage (not applicable to samples containing α-carotene, γ-carotene, zea-carotene, cryptoxanthin, and xanthophylls)	Spectrophotometric 436 nm	*J. Assoc. Off. Anal. Chem.*, 53, 181, 1970[128]
7. 45.1.04	Carotene Xanthophylls	AOAC Official Method 970.64 Carotene and xanthophylls in dried plant materials and mixed feeds	Spectrophotometric 436 nm	*J. Assoc. Off. Anal. Chem.*, 57, 511, 1974[129]
8. 45.1.34	Retinol	AOAC Official Method 2001.13 Vitamin A (retinol) in foods	LC 328 or 313 nm	None
9.	β-Carotene	AOAC Official Method 2005.07 β-Carotene in supplements and raw materials	LC 448 or 445 nm	*J. AOAC Int.*, 88, 1279, 2005;[130] 87, 1070, 2004[223]
American Association of Cereal Chemists, *Approved Methods*, vol. 2, 2000[131]				
1. AACC 86-01 A	Retinol	Vitamin A in dry vitamin mixes, beadlets, oils, and emulsions (>10,000 IU g^{-1})	Spectrophotometric 325 nm	None

Continued

Table 1.9 (Continued)

Source	Form	Methods and application	Approach	Most current cross-reference
American Association of Cereal Chemists, *Approved Methods*, vol. 2, 2000[131] (*Continued*)				
2. AACC 86-02	Retinol	Vitamin A in nonfat and instantized dry milks Carr-Price method	Colorimetric 620 nm	None
3. AACC 83-03	Retinol	Vitamin in enriched flour (free of carotenes) Carr-Price method	Colorimetric 620 nm	None
4. AACC 86-05	Retinol Carotene	Vitamin A and carotene in enriched cereals and mixed feeds Carr-Price Method	Colorimetric 620 nm (retinol) Spectrophotometric 440 nm (carotene)	*J. Assoc. Off. Anal. Chem.*, 41, 591, 1958[132]
5. AACC 86-06	Retinol and α-tocopherol	Vitamin A and vitamin E in foods by LC	LC, Fluorescence Ex λ = 290 Em λ = 330	*J. Agric. Food Chem.*, 25, 1127, 1977[133]
***Food Chemicals Codex*, 5th ed., 2004[134]**				
1. Page 90	β-Carotene	β-carotene (NLT 96.0%, NMT 101.0%)	Spectrophotometric 455 nm	None
2. Pages 429–430	Retinol Retinyl acetate Retinyl palmitate	Vitamin A (NLT 95.0%, NMT 100.5% of the declared vitamin A activity)	LC 325 nm	None
***Methods for the Determination of Vitamin in Foods*, COST 91, 1985[135]**				
1. Page 23	Retinol	Vitamin A in food saponification	LC 325 nm	*J. Assoc. Off. Anal. Chem.*, 66, 746, 1983[136]
2. Page 33	Total carotenes	Complex foods, fruits and vegetables, beverages, saponification, aluminum oxide clean up	Spectrophotometric 450 nm	*Z. Lebensm. Unters. Forsch.*, 163, 21, 1977[137]
European Committee for Standardization, 2000[273,274]				
1. EN 12823-1[273]	All-*trans*-retinol 13-*cis*-retinol	Foods	Saponification Normal phase LC on Si60 Spectrophotometric 325 nm Fluorometric Ex λ = 325 Em λ = 475	EN 12823-1[273]
2. EN 12823-2[274]	β-carotene	Foods	Saponification Reversed-phase LC on C_{18} either single column or in series Spectrophotometric 450 nm	EN 12823-2[274]

1.3.1 The Carr-Price colorimetric method

The most widely used assay for vitamin A until the advent of LC analysis in the 1970s was the Carr-Price colorimetric procedure. Methodology is given in AOAC Official Method 974.29 (45.1.02) "Vitamin A in Mixed Feeds, Premixes, and Foods, Colorimetric Method."[126] The chemistry is based on the formation of a blue complex between antimony trichloride or trifluoroacetic acid with retinol in chloroform, which is measured at 620 nm. The method is still used throughout the world, but should be replaced by LC methods in all situations unless instrumentation is not available to change to LC analysis. Often-mentioned deficiencies of the Carr-Price method include lack of specificity, unstable color that requires rapid and time-consistent measurement, and the use of corrosive and carcinogenic reagents. Procedural steps must be carefully controlled throughout the assay. Because of the extensive use of the Carr-Price method, problems associated with the procedure have been discussed in detail by Thiex and colleagues.[138] The Thiex and colleagues[138] summary, with its practicality of the treatment detailing sources of errors in the assay, and applicability to vitamin A assays in general, follows:

Systematic errors producing a positive bias

1. Presence of high levels of carotenoids or other interfering substances such as phenothiazine, or ethoxyquin
2. Solids remaining after hydrolysis that affect dilution volume
3. Less than complete recovery of standard at the partitioning step
4. Excessive standing of the standard solution in hexane after partitioning and before partitioning
5. Failure to remove standard from the evaporator immediately after hexane removal
6. Moisture in cuvettes
7. Cloudy solutions in cuvette
8. Use of degraded standard

Systematic errors producing a negative bias

1. Sample abuse including undue storage before analysis and exposure to heat, light, and oxidants
2. Grinding of mineral mix samples
3. Failure to completely solubilize vitamin during saponification
4. Failure to add antioxidant or stabilizer during hydrolysis
5. Failure to keep sample dispersed during hydrolysis
6. Use of old potassium hydroxide solution for saponification
7. Failure to use low actinic glassware and/or subdued lighting
8. Incomplete partitioning of retinol from the hydrolyzate
9. Inclusion of sodium sulfate or other particulates from digest when removing aliquotes
10. Poor-quality hexane
11. Failure to remove sample residue from the evaporator immediately after hexane evaporation
12. Moisture in pipettes or cuvettes
13. Standard concentration actually higher than the value used in calculation

Random errors producing poor precision

1. Lack of sample homogeneity
2. Failure to properly reduce samples
3. Too small sample size
4. Use of a single standard reading from a single day to make calculations
5. Use of an unstable spectrophotometer or detector

AOAC Method 974.29 permits use of trifluoroacetic acid in place of antimony trichloride. Thiex and colleagues[138] report that trifluoroacetic acid is easier to use, less corrosive, and provides a more stable color as compared to antimony trichloride.

1.3.2 Advances in vitamin A and carotenoid analysis

1.3.2.1 Spectroscopic methods

Various advanced spectroscopic methods are used to characterize and quantify carotenoids in model and biological systems. Recent applications include resonance Raman spectroscopy, ^{1}H-nuclear magnetic resonance spectroscopy, near-infrared spectroscopy, photoacoustic spectroscopy, transient lens spectroscopy, and electroabsorption spectroscopy. Applications of such sophisticated spectroscopic methods are provided in Table 1.10.[139–151] Pertaining to these applications, Raman spectroscopy has been widely adapted to measure carotenoids through nondestructive techniques in foods and tissues.

Raman spectroscopy with laser excitation defines highly specific vibrational and rotational energy levels of a molecule. When light irradiates a multimolecular system, the light is scattered both elastically and inelastically. The inelastic portion is the Raman scattered light. It shows a frequency shift of the excitational light, which corresponds to the vibrational/rotational energy transitions of the molecule bends with Raman peaks at 1525, 1159, and 1008 cm^{-1} respectively.[146]

1.3.2.2 High performance liquid chromatography

For routine assessment of vitamin A activity and for characterization of retinoids and carotenoids in biological samples, LC resolution is clearly the analyst's best analytical technique. Obvious advantages of LC over older approaches are the abilities to resolve *cis*-isomers from all-*trans*-retinol and all-*trans*-carotenoids, to resolve provitamin A carotenoids from complex carotenoid mixtures, and to use LC resolution as a preparatory step for characterization studies. Many of the reviews cited in Section 1.3 provide excellent overviews of LC applications, and Tables 1.11[152–184] and 1.12[185–244] provide details of analysis of different biological matrices. It is important to note that these references were selected from many excellent sources published over the last decade. Because of its accepted role as the primary analytical tool for retinoid and carotenoid analysis, the remainder of this chapter concentrates on LC and the combination of LC with mass spectrometry.

1.3.2.2.1 Extraction procedures for the analysis of retinoids and carotenoids by liquid chromatography. Saponification and direct solvent extraction are commonly used to extract retinoids and carotenoids from biological samples. Supercritical fluid extraction (SFE) has been less commonly applied. When applicable, direct solvent extraction is preferable to saponification because of time, lower solvent costs, and a better ability to avoid formation of artifacts in the extract. Artifact formation from the relatively unstable analytes (Section 1.2.2) can lead to underestimation of vitamin A activity, poor chromatographic resolution, and greater possibility of peak misidentification. It should always be kept in mind that all-*trans*-retinol and all-*trans*-carotenoids are more subject to degradation by isomerization and oxidation as the sample matrix is destroyed and the retinoids and carotenoids are extracted into the partitioning solvent. Rodriguez-Bernaldo de Quiros and Costa[122] provided a detailed review of extraction procedures covering saponification, solvent extraction, and SFE.

1.3.2.2.1.1 Saponification. Saponification is routinely used to extract retinol from tissues and animal-based foods and carotenoids from fruits, vegetables, and prepared foods. These matrices are often difficult to extract efficiently by direct solvent extraction owing to the inability of the solvent system to effectively penetrate the sample matrix, or the need to

Table 1.10 Application of Spectroscopic Methods to Analysis and Characterization of Carotenoids

Matrix	Description	Results	References
Raman spectroscopy			
Model systems Zeaxanthin Canthaxanthin Astaxanthin β-carotene	Semiempirical AM1 calculations were used to determine structures through quantum chemical calculations	The influence of hydroxy and keto groups on the structure of carotenoids revealed that the polyene chain was the sole source of the Raman spectra. Differences between carotenoids were due to the contribution of the carbonyl group to the π-electron system.	*Biospectroscopy*, 5, 19, 1999[139]
Human skin Γ-, β-, α-, δ-carotene, lycopene, lutein, zeaxanthin, phytoene, phytofluene	Use of Raman scattering to determine carotenoid concentration in normal and diseased human skin	Raman spectroscopy provided an accurate and precise method to noninvasively quantify carotenoids in human skin. Carotenoid concentrations correlated with the presence or absence of skin cancer or precancerous lesions.	*J. Invest. Dermatol.*, 115, 441, 2000[140]
Model system β-carotene	Photoinduced decomposition of all-*trans*-β-carotene in the absence of oxygen was studied by 2D Raman correlation spectroscopy	Intensity increase of the Raman band at 1537 cm^{-1} was postulated to be due to a product containing one or more *cis*-double bonds.	*J. Phys. Chem.* A, 106, 3371, 2002[141]
Model system β-carotene	Infrared and Raman spectroscopy were used to provide a computational study of the structure and energetic and vibrational spectroscopic characteristics of β-carotene	Calculations of electronic structure delineated the centrosymmetric structure of β-carotene. Higher stability of *cis*-isomers was determined to be due to an energetically favored β-ionone ring compared to all-*trans*-β-carotene.	*J. Raman Spectrosc.*, 34, 413, 2003[142]
Salmon muscle Astaxanthin Cantaxanthin	Raman spectroscopy was used to quantify fat and carotenoid content of Atlantic salmon muscle.	The method was quantitative at low ppm levels (1–10 $\mu g\ g^{-1}$).	*Appl. Spectrosc.*, 58, 395, 2004[143]
Human skin β-carotene Lycopene	Raman cross sectional profiles using two-wavelength excitation at 488 and 514.5 nm selectively quantified β-carotene and lycopene	The noninvasive method showed that β-carotene and lycopene levels in human skin measured on the inner palm vary from individual to individual. The method can be clinically applied to large population groups to estimate antioxidant status.	*J. Biomed. Optics*, 9, 332, 2004;[144] Laser Physics, 15, 295, 2005[145]
Fruit and vegetables β-carotene, lycopene, lutein, zeaxanthin	Raman response at 488 nm was used to assess carotenoid levels in fresh fruit and vegetables.	Raman spectroscopy provided a rapid screening method for measuring several carotenoids in fresh fruit and vegetables. Values compared to those obtained by LC.	*J. Agric. Food Chem.*, 52, 3281, 2004[146]

Continued

Table 1.10 (Continued)

Matrix	Description	Results	References
Raman spectroscopy (*Continued*)			
Spinach photosystem I particles all-*trans*- and 9-*cis* β-carotene, lutein, violaxanthin, 9-*cis*-neoxanthin	Low-temperature Raman spectroscopy provided precise information on the type and conformation of carotenoids in spinach Photosystem I particles	Spectral properties, binding sites and composition of major carotenoids were characterized.	*Biophys. Chem.*, 114, 129, 2005[147]
Other spectroscopic methods			
Near-infrared reflectance spectroscopy (NIRS)			
Maize α-,β-carotene, α-, β-cryptoxanthin, isolutein, lutein, violaxanthin, zeaxanthin	NIRS predicted carotenoid content through scores from modified partial least-square independent variables	The method quantified carotenoids in ground corn in minutes. Values compared to LC values.	*J. Agric. Food Chem.*, 52, 5577, 2004[148]
Photoacoustic and optical transmission spectroscopy			
Sesame oil β-carotene and geometric isomers	Photoacoustic spectroscopy was used to study linear polyene constituents in geometric isomers of β-carotene	Photoacoustic spectra accurately identified geometric isomers of β-carotene. Polyene systems within the β-carotene molecules were identified.	*Instrum. Sci. Technol.*, 33, 9, 2005[149]
Transient lens spectroscopy			
Model systems Seven C_{40} carotenoids	Transient lens pump-probe spectroscopy was used to characterize internal conversion dynamics ($S_2 \rightarrow S_1 \rightarrow S_0$) of carotenoids in various polar solvents	The extracted time constant (λ_1) of the internal conversion, $S_1 \rightarrow S_2$, showed a systematic increase with decreasing conjugation length.	*Phys. Chem. Chem. Phys.*, 7, 2793, 2005[150]
Electroabsorption spectroscopy			
Model systems β-carotene homologs	Optical nonlinear-polarizability of carotenoid homologs was measured by electroabsorption spectroscopy	Changes in polarizability ($\Delta\alpha$) and dipole moment ($\Delta\mu$) upon photoexcitation of β-carotene homologs with increasing number of conjugated double bonds were used to characterize optical nonlinearity and configuration of excited-state energies.	*Phys. Rev.*, 71, 195118, 2005[151]

Table 1.11 LC and LC-MS Methods for the Analysis of Retinol and β-Carotene in Foods, Pharmaceuticals, and Biologicals

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods					
Dried skim milk/ retinol	Saponify, reflux 30 min, extract w/PE, dilute w/PE after addition of BHT, and evaporate aliquot to dryness. Dissolve residue in MeOH	C_8 or C_{18}, 10 μm, 25 cm × 4.6 mm Mobile phase—isocratic H_2O:MeCN (10:90) Flow rate—2 mL min^{-1}	325 nm	—	*Int. Dairy Fed.*, 285, 53, 1993[152]
Infant formula/ retinol	Saponify, ambient, 18 h. Extract w/ Hex: Et_2O (85:15). Centrifuge, reextract. Add hexadecane to extract, evaporate aliquot, and redissolve in heptane	Apex silica, 3 μm, 15 cm × 4.5 mm Mobile phase—isocratic Heptane:IPA Flow rate—1–2 mL min^{-1}	324.5 nm	S_r—98.8 S_R—265.4 %RSD_r—3.6 %RSD_R—9.7	*J. Assoc. Off. Anal. Chem.*, 76, 399, 1993[127]
Danish calf, pig, ox liver/ all-*trans*-retinol, 13-*cis*-retinol	Saponify, extract w/Et_2O, IS-retinyl acetate, evaporate, redissolve in n-heptane, inject	LiChrosorb Si60, 25 cm × 4.6 mm, 5 μm Mobile phase—gradient IPA in n-heptane Flow rate—1.05 mL min^{-1}	325 nm	%Recovery—9.7	*J. Food Compos. Anal.*, 6, 3, 1993[153]
Cheese/ retinol	Homogenize in water:EtOH:Hex (8:20:20) containing 2% BHT. Add water, centrifuge. Remove Hex layer, dilute w/Hex	LiChrosorb Si60, 25 cm × 4.0 mm Mobile phase—isocratic Hex:IPA (99.8:0.2) Flow rate—2 mL min^{-1}	325 nm	%CV—2.6–8.8	*NAHRUNG*, 38, 527, 1994[154]
Milk/retinol	Saponify, 70 °C, 20 min. Extract w/heptane:DIPE (3:1), reextract. Inject extract	HS-5-Silica, 12.5 cm × 4.0 mm Mobile phase—gradient Heptane:IPA (60:1) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 344 Em λ = 472	QL <0.04 mg L^{-1} %Recovery—98.5 %CV—3.6	*J. Dairy Res.*, 61, 233, 1994[155]
Liver, liver products, infant foods/ retinol, 7-*cis*-, 9-*cis*-, 9,13,-di-*cis*-, 13-*cis*-, 11, 13-di-*cis*-, 11-*cis*-	Low fat—digest w/pancreatin at pH 9.0. High fat—mix w/water:EtOH (1:1). Saponify all samples, ambient, 16 h. Dilute, digest w/water and EtOH to give 1:1 ratio, pass aliquot through Kieselguhr column. Elute w/PE, evaporate, dissolve residue in iOCT	Spherisorb SW, 3 μm, 10 cm × 2 mm Mobile phase—isocratic iOCT:IPA (98.75:1.25) Flow rate—0.4 mL min^{-1}	Retinol 325 nm 13-*cis*-retinol 329 nm	—	*Z. Lebensm. Unters-Forsch.*, 199, 206, 1994;[156] *J. Chromatogr.* A, 693, 271, 1995[157]

Continued

Table 1.11 (Continued)

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods (*Continued*)					
Raw and home-prepared foods/retinol, β-carotene	Digest sample w/50 mL 2 N KOH for 30 min. Extract w/DIPE, after separation, saponify the upper layer w/5% KOH, then wash the upper layer w/10% NaCl. Wash the organic phase w/distilled water to alkaline free. Dry the sample and redissolve in mobile phase	Micro Pak MCH, 5 μm, 15 cm × 4 mm Mobile phase—MeOH: MeCN:A (30:60:10) for β-carotene ZorbaxODS, 5 μm, 25 cm × 4.6 mm Mobile phase—MeOH for retinol Flow rate—1.2 and 1.5 mL min^{-1} for β-carotene and retinol, respectively.	450 nm for β-carotene 325 nm for retinol	—	*Food Chem.*, 64, 163, 1999[158]
Plant, animal tissue, human serum/retinoids, carotenoids	Extract sample using A and Hex w/water	NP: Microsorb, 3 μm, silica, 10 cm × 3.6 mm. Mobile phase—isocratic Hex:IPA:HAC (100:0.5:0.1) Flow rate—1 mL min^{-1} RP: Microsorb, 3 μm, C_{18}, 10 cm × 3.6 mm Mobile phase—gradient MeCN-CH_2Cl_2-water-10 mM NH_4OAC	UV/Vis	%Recovery ≥ 95	*J. Chromatogr.* A, 936, 71, 2001[159]
Fortified fluid milk/retinyl palmitate	Add 5 mL EtOH to 2 mL milk in N_2-purged centrifuge tube, vortex, 30 s, stand, 5 min; add 5 mL Hex, vortex, 30 s, stand, 2 min; mix two times; add 3 mL water, vortex, 5 s; centrifuge, 10 min. Inject Hex layer	Unbonded Silica column, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic Conditioned Hex:IPA (99.85:0.15) Flow rate—1 mL min^{-1}	325 nm	$\%RSD_r$—1.5–5.7 $\%RSD_R$—5–22.7	*J. AOAC Int.*, 86, 375, 2003[160]
Cod liver oil, all-*trans*-retinol, 13-*cis*-retinol	Saponify, extract w/Hex:CH_2Cl_2	Zorbax SIL Mobile phase—isocratic Hex:CH_2Cl_2:IPA (300:200:1.5) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 365 Em λ = 510	—	*J. Agric. Food Chem.*, 25, 1127, 1977[161]
LC-MS					
Fish eggs/all-*trans*-retinol, carotenoids	Extract samples w/A 3×, centrifuge. Extract combined extract w/MTBE and water 4×. Evaporate the combined MTBE phase under low pressure, 20°C. filter.	Finesse Genesis C_{18}, 4 μm, 15 cm × 2.1 mm Mobile phase—gradient MeOH–MTBE–water Flow rate—0.2 mL min^{-1}	MS/MS-ESI Positive ion mode w/MRM	QL—4.7–7.7 p. μL^{-1} (injected)	*J. Chromatogr.* B, 816, 49, 2005[162]

Pharmaceuticals					
Dietary supplements/ retinol, β-carotene	Hex—CH_2Cl_2 extract for soft-gel capsules. Treat tablet form w/EtOH and CH_2Cl_2 following addition of hot water (55°C)	C_{18} Econosphere column, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient MeOH–IPA Gradient flow rate—1–1.5 mL min^{-1}	UV/VIS 325 nm and 450 nm	%Recovery—90–116	*J. AOAC Int.*, 85, 1127, 2002[163]
Biologicals					
Blood/retinol, retinoic acid, retinoyl-β-glucuronide	Add EtOH and EtOAC, centrifuge, wash pellet w/EtOAC, add water to supernatant to separate phase. Add HAC to aqueous phase, reextract w/EtOAC, wash pooled EtOAC extract. Dry over Na_2SO_4, evaporate under argon, and dissolve residue in MeOH	Resolve C_{18}, 5 μm, 15 cm × 3.9 mm Mobile phase—isocratic MeOH:water (7:3) containing 10 mM NH_4OAC Flow rate—1.5 mL min^{-1}	355 nm	—	*Am. J. Clin. Nutr.*, 43, 481, 1986[164]
Plasma, amniotic fluid, embryo/retinol, retinoic acid, 13-*cis*-retinoic acid, 4-oxo-metabolites	Add IPA, freeze overnight in liquid N_2, centrifuge. Inject directly	Spherisorb 3 ODS II, 3 μm, 12.5 cm × 4.6 mm Mobile phase—gradient 40 mM NH_4OAC–MeOH Flow rate—2 mL min^{-1}	354 nm	%Recovery—90–100	*J. Liq. Chromatogr.*, 11, 2051, 1988;[165] *Reprod. Toxicol.*, 7, 11, 1993[166]
Biological fluids/retinoic acid, 13-*cis*-retinoic acid, 4-oxo-metabolites	Add Ro 11-6738 (3) or etretin (IS) in water:MeCN (8:2). Centrifuge. Online solid phase extraction	Two Spherisorb ODS 1, 5 μm, in series Mobile phase—gradient MeCN–NH_4OAC–HAC Flow rate—1 mL min^{-1}	360 nm	QL—0.3 ng mL^{-1} %Recovery—63–98 %CV <5	*J. Chromatogr.*, 424, 303, 1988[167] & 593, 55, 1992;[169] *Meth. Enzymol.*, 189, 146, 1990[168]
Plasma/retinol, retinoic acid, 13-*cis*-retinoic acid	Add arotinoid ethyl sulfonic acid (Ro15-1570) (IS) in EtOH, vortex, add water and Hex. Acidify w/2 M HCl. Centrifuge, evaporate organic layer. Dissolve residue in mobile phase	Spherisorb S5W silica, 5 μm, 15 cm × 4.6 mm Mobile phase—isocratic Hex:IPA:HAC (200:0.7:0.135) Flow rate—0.9 mL min^{-1}	350 nm	%Recovery >83	*Clin. Chem.*, 40, 48, 1994[170]

Continued

Table 1.11 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Biologicals (*Continued*)					
Serum/retinoic acid, *cis*-retinoic acid	Dilute serum w/10 × volume of MeCN: 100 mM NH_4OAC (1:3), pH 5.5. Extract w/Hex, concentrate organic phase, evaporate under N_2, dissolve residue in MeOH:MeCN (1:2), centrifuge.	Chemcosorb 5-ODS-H, 5 µm, 15 cm × 4.6 mm Mobile phase—isocratic MeCN:MeOH (2:1):NH_4OAC, pH 7.0 Flow rate—1 mL min^{-1}	340 nm	%RSD—3.7–14.2 %Recovery—79–89	*J. Chromatogr. B*, 657, 53, 1994[171]
Serum/ retinoic acid, 13-*cis*-retinoic acid, 4-oxo-matabolites	Add RO 13-6307 (IS) and 1 M HAC, transfer to Bakerbond SPE octadecyl column equilibrated w/MeOH and HAC. Wash w/A:1 M HAC (1:1), dry under vacuum. Elute w/MeCN, evaporate. Dissolve residue in mobile phase	LiChrosorb Si60, 5 µm, 25 cm × 4 mm Mobile phase—isocratic CH_2Cl_2:1,4-dioxane (180:40) containing 1% HAC Flow rate—0.8 mL min^{-1}	360 nm	%CV—7.2–12.4	*J. Chromatogr. B*, 666, 55, 1995[172]
Human liver/ retinol, retinyl esters	Homogenize in EtOH containing 1% pyrogallol, shake 30 min, 4°C. Add water, extract 2 × w/Hex. Centrifuge, evaporate, dissolve residue in MeOH spiked w/retinyl acetate, saponify, 30 min, 60°C, to determine total retinol	Supelosil LC-8, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:water (94:6) Flow rate—1.5 mL min^{-1}	PDA 325 nm	%Recovery—80–100 %CV < 11	*J. Chromatogr. B*, , 668, 233, 1995[173]
Bovine serum/ retinol, retinaldehyde, retinoic acid, 13-*cis*-retinoic acid	Add MeCN and AA (0.02 M), centrifuge. Add water to make MeCN:water (4:6)	ODS-AQ YMC, 3 µm, 150 × 50 µm and 360 × 180 µm Mobile phase—isocratic MeCN:water:MeOH (65:32.5:2.5) containing 1% TBAP, pH 5.0 Flow rate—4 µL min^{-1}	EC Ag/AgCl + 0.9 V	DL (on-column)—0.27–2.7 fmol	*J. Chromatogr. B*, 677, 225, 1996[174]
Mouse, plasma, embryo/ retinoic acid, 4-oxo, 5,6-epoxy-, 9-*cis*-, 13-*cis*-	Add 4-(5,6,7,8-tetrahydro-5,5,8,8 tetramethyl-2-anthracenyl) benzoic acid (IS) in MeOH. Add MeCN, shake, centrifuge, evaporate to dryness. Dissolve residue in mobile phase	Spherisorb ODS-2, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:water:MeOH:*n*-butyl alcohol (56:37:4:3) containing 10 mM NH_4OAC and 70 mM HAC Flow rate—1 mL min^{-1}	325 nm	%CV—3~9	*J. Chromatogr. B*, 681, 153, 1996[175]

Plasma/ retinol, retinoic acid, 4-oxo-retinoic acid, 13-*cis*-4-oxo, 13-*cis*-retinoic acid, 9-*cis*-retinoic acid	Add IPA (3 × volume) containing 0.012% BHT, vortex, centrifuge. Dilute 0.4 mL w/1.2 mL 2% NH_4OAC. Clean up—AASPC2 cartridge	Spherisorb ODS, 3 μm Mobile phase—gradient 60 mM NH_4OAC–MeOH–IPA Flow rate—1 mL min^{-1}	UV 340 nm 356 nm	—	*Life Sci.*, 59, PL169, 1996[176]
Sea urchin/ carotenoids, retinol	Retinol: saponify the sample w/KOH and extract w/Hex. Carotenoids: mix sample w/$CaCO_3$, washed sea sand and anhydrous Na_2SO_4. Extract w/A:PE (1:1) till residue was colorless, vaccum filter each time Evaporate an aliquot of the extract to dryness, redissolve in HPLC eluent	Carotenoids: Sperisorb ODS, 25 μm, 25 cm × 4.6 mm. Mobile phase—gradient MeOH–MeCN–CH_2Cl_2–Hex Flow rate—0.8 mL min^{-1} Retinol: Kromasil 100 C_{18}, 5 μm, 25 cm × 4 mm. Mobile phase—isocratic MeOH:MeCN (1:1) Flow rate—1 mL min^{-1}	Carotenoids: 450 nm Retinol: Fluorescence Ex λ = 297 Em λ = 327	RPT—0.4–2.6% RPD—0.4–6.7% %Recovery > 93 DL carotenoids—16–128 ng mL^{-1} retinol—1.35 μg mL^{-1}	*Eur. Food Res. Technol.*, 212, 687, 2001[177]
Bovine serum/ retinol, retinoic acid, retinyl palmitate	Add 2.1 mL MeCN and 0.1 mL 2 N HAC to 2 mL sample, vortex. Extract w/1.0 mL water and 7.5 mL Hex:IPA (6.5:1.5), vortex, 2 min. Centrifuge, evaporate the organic layer. Redissolve residue in 0.2 mL mobile phase	Symmetry C_{18}, 5 μm, 15 cm × 3 mm Mobile phase—gradient MeOH–water–10 mM NH_4OAC–HAC–CH_2Cl_2 Flow rate—0.4 mL min^{-1}	350 nm	QL—1–86 ng mL^{-1} $\%CV_{Intra}$—4.5–20.7 $\%CV_{Inter}$—9.4–22 %Recovery—57–73	*Anal. Chim. Acta*, 468, 237, 2002[178]
Ram plasma/ retinoic acid, retinol, retinyl palmitate	Add three volume of IPA:CH_2Cl_2 (2:1) to sample, vortex, centrifuge. Evaporate supernatant to near dryness. Redissolve residue in THF: MeOH (1:19)	Hypersil MOS C_8, 5 μm, 25 cm × 4 mm Mobile phase—gradient MeOH–NaOAC–HAC–water–THF Flow rate—1 mL min^{-1}	350 nm for retinoic acid and 325 nm for the others	DL—0.3–10 ng mL^{-1} %RSD—1.2–6.2 %Recovery—74–90	*J. Liq. Chromatogr. Related Technol.*, 26, 559, 2003[179]
Human serum, rat liver/ retinol, β-carotene	Mix sample w/water–EtOH, incubate, 70°C, 5 min. Saponify the mixture w/10 M KOH, 70°C, 30 min. Extract w/Hex, 4×. Evaporate the combined extract to dryness, redissolve in mobile phase	C_{18}, 5 μm, 15 cm × 3.9 mm Mobile phase—isocratic MeCN:CH_2Cl_2:MeOH–IPA (90:15:10:0.1) Flow rate—1 mL min^{-1}	UV/VIS 325 nm 451 nm	%Recovery—92.8–107.1 $\%CV_{Intra}$—4.7–7.1 $\%CV_{Inter}$—2.7–6.4	*J. Chromatogr. B*, 791, 305, 2003[184]

Continued

Table 1.11 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
LC-MS					
Plasma/retinoic acid, 13-*cis*-retinoic acid	Add IPA containing 0.01 M BHT, vortex and centrifuge. Add 1% NH_4OAC to supernatant. Apply to Methyl-C_1 Accubond SPE, elute retinoids w/MeCN. Derivitatize to pentafluorobenzyl esters, dry, dissolve in MeCN (0.01 M BHT)	Nova-Pak C_{18}, 15 cm × 3.9 mm and 7.5 cm × 3.9 mm Mobile phase—gradient MeCN–0.1 M NH_4OAC, pH 5.0	PDA 369 nm or MS-APCI Negative ion mode w/SIM	DL (on-column)—0.25 ng	*J. Pharmaceut. Sci.*, 85, 287, 1996[180]
Standards/ retinol, retinaldehyde, retinoic acid, retinyl acetate	—	YMC C_{30}, 25 cm × 4.6 mm Mobile phase—gradient MeOH–water–HAC–*tert*-butyl ether Flow rate—0.6 mL min^{-1}	MS–ESI Negative, positive ion mode for retinoic acid	DL (on-column)—0.5 ng, 23 p.	*FASEB*, 10, 1098, 1996[181]
Serum/retinol, β-carotene	Mix serum sample (0.2 mL for retinol, 1.0 mL for β-carotene) w/1 mL 30% NaCl and 1 mL 70% EtOH and then extract 3x w/3 mL Hex. Evaporate combined Hex extracts, dissolve residue in 200 μL MeOH:MTBE (1:1)	YMC C_{30}, 3 μm, 25 cm × 4.6 mm for β-carotene; 10 cm × 2.1 mm for retinol Mobile phase—gradient MeOH (containing 1 mM NaOAC)–MTBE for β-carotene MeOH–MTBE–HAC–water for retinol. Flow rate—1 mL min^{-1}	MS-APCI positive ion mode w/SIM	QL (on column) <1 pmol	*Anal. Chem.*, 72, 4999, 2000[182]
Rat prostate/ all-*trans*–, 9-*cis*, 13-*cis*-retinoic acid, retinol	Homogenize sample w/water on ice. Add three volumes ice-cold EtOH, vortex, centrifuge. Wash precipitate w/one volume icecold EtOH, centrifuge. Evaporate combined supernatant to dryness and dissolve in 0.5 mL icecold EtOH, sonicate, centrifuge, and evaporate supernatant to dryness. Redissolve residue in weak mobile phase	Micra nonporous silica C_{18}, 33 × 4.6 mm Mobile phase—gradient MeOH–water–HAC–MTBE Flow rate—0.25 mL min^{-1}	MS-APCI Positive ion mode w/SIM	QL all-*trans*-retinoic—702 fM acid retinol—2.14 pM %CV—6.4–6.7 %Recovery >86	*J. Mass Spectrom.*, 36, 882, 2001[183]

Table 1.12 LC and LC-MS Methods for the Analysis of β-Carotene and Other Carotenoids in Foods and Biologicals

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods					
Carrots, green vegetables/ β-carotene, 15,15′-*cis*-β-carotene	Add decapreno-β-carotene (IS) and β-apo-8′-carotenal (IS) and Na_2SO_4 plus $MgCO_3$. Extract w/THF, reextract. Evaporate, extract residue w/PE, dry over Na_2SO_4, evaporate. Dissolve residue in Hex	Microsorb C_{18}, 5µm, 25 cm × 4.6 mm Mobile phase Isocratic for β-carotene and 15,15′- *cis* isomer; Gradient for xanthophylls and β-apo-8′-carotenal	PDA 446–450 nm, 500 nm	—	*J. Chromatogr.*, 346, 237, 1985[185] *J. Agric. Food Chem.*, 34, 603, 1986[186]
Fruits and vegetables/α–, β-carotene, 9-*cis*- and 13-*cis*-isomers	Puree, mix w/MeOH. Filter, extract filter cake w/A:Hex (1:1). Extract A and MeOH w/water, saponify epilayer, ambient, 30 min. Wash w/H_2O to remove bias. Dry over Na_2SO_4; filter	$Ca(OH)_2$, 115 mesh, 25 cm × 4.6 mm Mobile phase—isocratic A:Hex (3:97) Flow rate—0.9 mL min^{-1}	436 nm 340 nm	%Recovery—90–95 DL (on-column)—20 µg	*J. Food Sci.*, 52, 669, 1987[187]
Yellow/orange vegetables/α-, β-, δ-carotene, 15,15′-*cis*-β-carotene, *cis*-δ-carotene	Add nonapreno-β-carotene (IS), extract w/mobile phase w/Na_2SO_4 and $MgCO_3$. Extract w/THF, filter, reextract until filtrate is colorless. Rotovap to remove most of solvent. Partition into PE:water (1:1). Wash water layer w/PE (multiple times), dry over Na_2SO_4, and evaporate. To evaluate presence of carotenol esters, ether extract were saponified, ambient, 3 h	Brownlee RP-18, 5 µm, 22 cm × 4.6 mm Mobile phase—isocratic MeOH:MeCN:CH_2Cl_2 (22:55:23) Flow rate—1 mL min^{-1}	PDA Lycopene, α-, β-carotene, 475 nm	%Recovery—98	*J. Agric. Food Chem.*, 35, 732, 1987[188]
Squash/33 analytes xanthophylls, carotenol esters, carotenoids, carotenol *cis* esters	Add isozeaxanthin (IS) and β-apo-8′-carotenol. Extraction—see the previous (yellow/orange vegetables)	Microsorb C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—gradient MeCN–MeOH–CH_2Cl_2–Hex Flow rate—0.7 mL min^{-1}	PDA 475 nm for lycopene, α-, β-carotene IS 402 nm	%Recovery >98	*J. Agric. Food Chem.*, 36, 929, 1988[189] & 36, 938, 1988[190]

Continued

Table 1.12 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods (*Continued*)					
Apricots, peaches, pink grapefruit, tomatoes, green, vegetables/ xanthophylls, carotenoids, carotenol esters	Extraction—see the previous (yellow/ orange vegetables)	Microsorb C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient MeCN–MeOH–CH_2Cl_2–Hex Vydac 201 TP54 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:MeOH:CH_2Cl_2:Hex (10:85:2.5:2.5)	PDA	—	*J. Agric. Food Chem.*, 37, 1465, 1989[191] & 40, 390, 1992[192]
Vegetables, fruits, berries/α-, β-, γ-carotene, lutein, lycopene	Extract w/A w/addition of Na_2SO_4 and $MgCO_3$, filter, rotovap. Saponify residue, ambient, overnight, w/BHT added. Extract w/Hex:Et_2O (7:3) containing 0.1% BHT	Zorbax ODS, 5–6 mm, or spherisorb ODS2, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic (Zorbax) MeCN:CH_2Cl_2:MeOH (70:20:10) Flow rate—2 mL min^{-1} Mobile phase—gradient (Spherisorb) MeCN–MeOH–CH_2Cl_2–Hex Flow rate—1 mL min^{-1}	PDA 450 nm	%Recovery—101–103	*J. Agric. Food Chem.*, 37, 655, 1989[193]
Fruits, sweet potato/ α-, β- carotene, β-cryptoxanthin	Saponify, ambient, overnight. Filter, extract w/Hex containing 0.01% BHT. Wash Hex w/water saturated w/NaCl. Filter through Na_2SO_4, evaporate, dissolve in mobile phase	Zorbax ODS or Vydac C_{18} 218TP54, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic Zorbax—MeCN:CH_2Cl_2:MeOH (35:15:0.1) containing 0.001% TEA Vydac—MeOH:MeCN:THF (28:25:2) Flow rate—1 mL min^{-1}	PDA 436 nm	%Recovery—70–100 QL—1 μg 100 g^{-1}	*J. Food Compos., Anal.*, 3, 119, 1990[194] & 6, 336, 1993[195]

Vegetables/α-, β-, γ-carotene, lutein, zeaxanthin, β-crytoxanthin, phytoene, phytofluene	Add retinyl palmitate (IS). Blend w/Na_2SO_4, $MgCO_3$ and THF, 2 ×, concentrate, rotovap, 40°C. Partition THF extract w/PE, evaporate to dryness. System I Dissolve residue in THF. System II Dissolve residue in THF:EtOH (1:3)	Spherisorb-S RP-18 or Spheri-ODS, 25 cm × 4.6 mm Mobile phase: System I—isocratic MeCN:CH_2Cl_2:MeOH (70:20:10) Flow rate—1.8 mL min^{-1} System—flow rate gradient MeCN:MeOH (85:15)	Carotenoids 450 nm Phytoene 286 nm Phytofluene 370 nm	—	*J. Agric. Food Chem.*, 40, 2135, 1992[196]
Red peppers/ 14 carotenoids	Add β-apo-8′-carotenal (IS), extract w/A. Extract A w/Et_2O, 10% NaCl solution. Dry w/Na_2SO_4, concentrate. If required, saponify residue or Et_2O extract, ambient, 1h	Spherisorb ODS 2, 5 μm, 25 cm × 4 mm Mobile phase—isocratic MeCN:IPA:water (39:57:4) or MeCN:IPA:MeOH:water (39:52:6:4) Flow rate—0.9–1.5 mL min^{-1}	PDA Multiple λ	—	*J. Agric. Food Chem.*, 41, 1616, 1993[197]
Carrots/α-, β-carotene, lutein	Extract w/A, filter. Reextract 2×. Evaporate, add NaCl saturated water and B: Et_2O (1:1). Collect organic layer, wash, dry over Na_2SO_4. Evaporate, dissolve residue in $CHCl_3$, dilute w/mobile phase	LiChrosorb ODS, 6 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:IPA:MeOH:water (39:52:5:4)	PDA Multiple λ	—	*J. Agric. Food Chem.*, 43, 589, 1995[198]
Tomato juice/ 11 carotenoids	Add β-apo-8′-carotenal (IS). Blend w/$MgCO_3$, celite, and THF. Filter, reextract until colorless. Reduce volume, partition w/CH_2Cl_2 and NaCl saturated water. Dry over Na_2SO_4. Reduce volume to 10 mL, filter	Microsorb MV C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:MeOH:CH_2Cl_2:Hex (40:20:20:20)	PDA Multiple λ	—	*J. Agric. Food Chem.*, 43, 579, 1995[199]
Mango/more than eight carotenoids	Extract w/cold A. Partition w/Et_2O: PE (1:1). Saponify, ambient, overnight. Wash organic layer free of base. Concentrate. Add Sudan I (IS) to aliquot, evaporate, and dissolve in Hex	Spherisorb Nitrile, 5 μm, 15 cm × 4.6 mm Mobile phase—Multilinear gradient A in Hex Flow rate—1 mL min^{-1}	PDA 400–500 nm identification by mass spectrometry	—	*J. Agric. Food Chem.*, 45, 120, 1997[200]

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Table 1.12 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods (*Continued*)					
Fruits and vegetables/α-, β-carotene, lutein, lycopene, β-cryptoxanthin, zeaxanthin	Extract freeze-dried sample w/MeOH: THF (1:1) until colorless, centrifuge after each extract. Combine extracts, extract w/PE until PE extract are colorless. Evaporate to dryness. Dissolve residue in MeOH:THF (75:25)	Vydac 201TP 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:THF (95:5) Flow rate—1 mL min^{-1}	450 nm	DL—1.0 mg mL^{-1} %RSD_r—1.9–4.9 %Recovery—93–107	*Food Chem.*, 59, 599, 1997[201]
Marigold flower extract/ carotenoids	Saponify the sample in 70 °C, 1 h, w/ethyl ether—15% KOH in MeOH. Wash mixture w/water till the pH at neutral point. Extract the mixture w/ethyl ether till colorless. Pass the combined extract through Na_2SO_4, evaporate the extract to dryness. Redissolve to desired volume w/mobile phase	YMC SB-SIL 200 C_{30}, 3 μm Mobile phase—isocratic MTBE–MeOH (3:97) Flow rate—1.0 mL min^{-1}	450 nm	—	*J. Agric. Food Chem.*, 47, 4189, 1999[202]
Orange juice/ lutein, zeaxanthin, α-, β-carotene, β-cryptoxanthin	Extract 5 mL sample w/EtOAC 3×50 mL w/BHT, add MeOH and 1 M NaCl to the combined the organic phase, mix and extract w/EtOAC again. Evaporate pooled EtOAC to dryness. Redissolve in mobile phase w/internal standard	Vydac 201 TP54 C_{18}, 5 μ, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:MeCN:$C_2H_4Cl_2$ (60:35:5) containing 0.05 M NH_4AC, 0.1% BHT and TEA. Flow rate—1.0 mL min^{-1}	450 nm	DL—0.01–0.02 mg L^{-1} %Recovery—61.3–78.1	*Food Chem.*, 64, 269, 1999[203]
Carrot juices, vitamin supplement drinks/carotene stereoisomers	Extract samples w/A:Hex (1:1), add 10% NaCl solution to remove the emulsion. Wash Hex layer w/water. Evaporate the extract to dryness, redissolve in IPA	YMC, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient MeOH–MTBE–water Flow rate—1 mL min^{-1}	PDA 452 nm, 445 nm, 471 nm and 462 nm	—	*Food Chem.*, 70, 403, 2000[204]
Leafy vegetables, cooked green vegetables/ 10 carotenoids	Extract sample w/cold A using a mortar and pestle, partition to PE. Saponifying when necessary. Evaporate extract to dryness under N_2, ≤35°C, dissolve residue in A.	Spherisorb S3 ODS2, C_{18}, 3μm, 15 cm × 4.6 mm Mobile phase—gradient MeCN–MeOH–EtOAC (0.05% TEA) Flow rate—0.5 mL min^{-1}	PDA 438 nm, 439 nm, 441 nm, 447 nm, 454 nm	—	*Food Chem.*, 78, 389, 2002[205] & 83, 595, 2003[206]

Tomato juice/ 16 carotenoids	Add $MgCO_3$ to the sample and extract w/EtOH:Hex (4:3). Collect upper layer, re-extract residue again. Extract the residue w/Hex, filter. Partition combined extracts and filtrate w/water and 10% NaCl. Evaporate to dryness under vacuum. Redissolve in CH_2Cl_2	YMC, C_{30}, 5μm, 25 cm × 4.6 mm Mobile phase—gradient Isobutanol–MeCN–CH_2Cl_2 Flow rate—2 mL min^{-1}	PDA 476 nm	DL—0.09–0.64 μg g^{-1} QL—0.27–1.98 μg g^{-1}	*J. Chromatogr. A*, 1012, 103, 2003[207]
Teas/38 carotenoids	Extract sample either cold A or hot water, and filter	Waters symmetry, C_8, 15 cm × 4.6 mm Mobile phase—gradient MeOH–MeCN–A–0.25 M pyridine Flow rate—1 mL min^{-1}	PDA 340–740 nm	—	*J. Agric. Food Chem.*, 51, 5307, 2003[208]
Cashew apple/ carotenoids	Extract w/cold A. Saponify, ambient, overnight. Wash organic layer free of base. Concentrate	Vydac C_{18} 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic 100% MeOH Flow rate—1 mL min^{-1}	PDA 250–600 nm	—	*Food Chem.*, 81, 495, 2003[209]
Various carrots/ α-, β-carotene, lutein, lycopene	Add IS and 2.5 g Na_2SO_4 to 0.5 g sample, extract sample w/CH_2Cl_2 and A. Dry the extract under argon and redissolve in CH_2Cl_2:MeOH (50:50)	Waters Resolve, C_{18}, 5 μm, 30 cm × 3.9 mm Mobile phase—gradient MeCN-Water-MeOH-CH_2Cl_2-NH_4OAC Flow rate—2 mL min^{-1}	PDA 444 nm, 453 nm, 445 nm, 472 nm for α-, β-carotene, lutein, lycopene, respectively.	%Recovery—64–105	*J. Agric. Food Chem.*, 52, 3417, 2004[210]
Cereals/ carotenoids	Saponify the sample, extract the suspension twice w/Hex:EtOAC (9:1). Evaporate the combined organic layer to dryness, redissolve in mobile phase	Kromasil Phenomenex Si, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic IPA:Hex (5:10) Flow rate—1.5 mL min^{-1}	PDA 450 nm	%Recovery—92–122	*J. Agric. Food Chem.*, 52, 6373, 2004[211]
Fruit and vegetable juices/ 17 carotenoids	Extract sample w/Hex:EtOH (3:4) and saponify suspension w/ET_2O, methanolic KOH, 0.1%. Extract w/ET_2O and 10% NaCl. Evaporate the extract to dryness, redissolve in ET_2O	Vydac 201TP54, C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient MeOH–0.1 M NH_4OAC–TBME–water	PDA 290 nm and 450 nm	%RSD—2.3–10.7	*J. Agric. Food Chem.*, 52, 2203, 2004[212]

Continued

Table 1.12 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods (*Continued*)					
Chinese medical herb/25 carotenoids	Extract sample w/Hex-EtOH-A-toluene, and saponify suspension w/40% KOH, 16 h. Extract w/Hex, add 10% Na_2SO_4, collect upper layer, reextract residue one more time. Evaporate combined extract to dryness, dissolve in mobile phase	YMC, C_{30}, 5 µm, 25 cm × 4.6 mm Mobile phase—gradient MeOH–IPA–CH_2Cl_2 Flow rate—1 mL min^{-1}	PDA 450 nm	—	*Chromato-graphia*, 60, 411, 2004[213]
Indian leafy/ β-carotene, lutein, neoxanthin, violaxanthin, zeaxanthin	Extract sample w/cold A till colorless. Mix the combined extracts w/Hex, 3×. Filter and dry the extract over anhydrous Na_2SO_4, evaporate the extract to dryness. Redissolve in Hex	SGE, C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:MeOH:CH_2Cl_2 (60:20:20) containing 0.1% NH_4OAC Flow rate—1 mL min^{-1}	450 nm	—	*J. Agric. Food Chem.*, 53, 2838, 2005[214]
Spear shrimp shells/ carotenoids	First extract sample w/A, 2×. Then extract reside w/PE, 2×. Mix combined extracts w/10% Na_2SO_4, collect supernatant, reextract residue w/PE, 4×. Evaporate all combined extracts to dryness. Redissolve in MeOH:CH_2Cl_2	Cosmosil Co, C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—gradient MeOH–MeCN–CH_2Cl_2–Water Flow rate—2 mL min^{-1}	PDA 480 nm	—	*J. Agric. Food Chem.*, 53, 5144, 2005[215]
Vegetables/ β-carotene, lycopene	Extract with Hex/A/EtOH (50:25:25), evaporate the layer, dissolve dry residue in MeOH/THF/MeCN (55:15:30)	µBondapak, C_{18}, 10 µm, 30 cm × 3.9 mm Mobile phase—isocratic MeOH–MeCN + 9 µM TEA Flow rate—0.9 mL min^{-1}	475 mm	%RSD—10.5	*Food Chem.*, 95, 328, 2006[216]
LC-MS					
Marigold, fruits/ lutein mono-and diesters	Marigold Grind, extract (2×) with MeOH:EtOAC:light petroleum (1:1:1). Dry, evaporate to dryness. Dissolve residue in light petroleum. Fruits Homogenize extract 3× with above extractant. Dry, evaporate to dryness. Dissolve residue in light petroleum	YMC—Pack 30, 5 µm, 25 cm × 4.6 mm Mobile phase—gradient TBME–MeOH–water Flow rate—1 mL min^{-1}	MS-APCI positive ion mode	—	*J. Agric. Food Chem.*, 50, 66, 2002[217]

Various plants/ zeaxanthin esters	Extract with MeOH:EtOAC:petroleum ether (1:1:1). Dry over anhydrous sodium sulfate and evaporate to dryness. Dissolve residue in TBME/ MeOH (1:1)	YMC—Pack C_{30}, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient TMBE–MeOH–water Flow rate—1 mL min^{-1}	MS-APCI positive ion mode	—	*J. Agric. Food Chem.*, 51, 7044, 2003[218]
Mandarin essential oil/carotenoid sters	Dilute oil 1:1 with MTBE containing 0.1% BHT	YMC—Pack C_{30}, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient MeOH–MTBE Flow rate—1 mL min^{-1}	MS-APCI Positive ion mode	—	*Flavor and Fragrance J.*, 21, 319, 2006[219]
Tropical fruits/ 11 carotenoids	Extract sample w/cold A, partition w/PE, saponify the suspension w/10% KOH. Evaporate the extract, and redissolve in acetone	Spherisorb ODS2, 3 μm, 15 cm × 4.6 mm Mobile phase—gradient MeOH (containing 0.5% TEA)–MeOH–EtOAC Flow rate—0.5 mL min^{-1}	PDA or MS	—	*J. Food Compos. Anal.*, 17, 385, 2004[220]
Red pepper pods/ carotenoids	Digest sample using enzyme, extract samples w/A:Hex (1:1), add 10% NaCl solution to remove the emulsion. Wash Hex layer w/water. Reextract aqueous phase w/EtOAC, Evaporate the combined extracts to dryness, and redissolve in IPA	YMC C_{30}, 3 μm, 15 cm × 3.0 mm Mobile phase—gradient MeOH–MTBE–water Flow rate—0.42 mL min^{-1}	MS/MS-APCI positive and negative ion mode	—	*Rapid Commun. Mass Spectrom.*, 19, 2617, 2005[221]
Vegetables, fruits/ carotenoids	Extract sample w/THF:MeOH (1:1), filter and reextract again. Extract combined filtrate w/PE, 2×. Evaporate combined extracts to dryness. Redissolve in initial mobile phase	Symmetry C_{18}, 3.5 μm, 75 × 4.6 mm and Atlantic dC_{18} 5 μm, 15 cm × 2.0 mm Mobile phase—isocratic MeOH:THF:MeCN (60:30:10)	MS-APCI positive mode w/SIM	—	*J. Agric. Food Chem.*, 53, 7371, 2005[222]
Pharmaceuticals					
Supplements, raw materials/ all-*trans*-, 9-*cis*-, 13-*cis*-, 15-*cis*-β-carotene	Water-dispersible formulations—enzymatically digest samples w/protease and extract w/CH_2Cl_2–EtOH Oily suspensions—directly dissolve sample in CH_2Cl_2–EtOH	YMC-Pack C_{30}, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic 0.01% AA–MeOH–0.01% TBME Flow rate—0.9 mL min^{-1}	445 nm	%RSD—1.2–4.4 %Recovery—98–102	*J. AOAC Int.*, 87, 1070, 2004[223]

Continued

Table 1.12 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Pharmaceuticals (*Continued*)					
Supplements, raw materials/all *trans*-β-carotene	*Oily solutions, suspensions* Dissolve in CH_2Cl_2 and EtOH containing BHT. *Powders, beadlets, emulsions* Digest with alkaline Protease R, extract with CH_2Cl_2 and EtOH containing BHT. *Tablets, capsules* Grind, digest with alkaline Protease R, extract with CH_2Cl_2 and EtOH containing BHT	System A Suplex Pkb-100, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic Propanol–*N*-ethyldiisopropylamine–NH_4OAC–MeCN–MeOH, BHT Flow rate—0.6 mL min^{-1} System B YMC—Pack C_{30}, 5 μm, 25 cm × 4.6 mm Mobile phase—step gradient MTBE–MeOH MTBE, ascorbic acid Flow rate—0.9 mL min^{-1}	System A 448 nm System B 445 nm	%RSD_R—3.35–23.09 HORRAT value—1.06–3.72	*J. AOAC Int.*, 88, 1279, 2005[130]
Biologicals					
Plasma/six carotenoids	Add echinenone (IS) in EtOH, add Hex. Vortex, centrifuge. Evaporate Hex phase. Dissolve in mobile phase	LC C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:CH_2Cl_2:MeOH (70:20:10) Flow rate—1.7 mL min^{-1}	PDA 436 nm	%CV—1.9–7.1	*J. Liq. Chromatogr.*, 8, 473, 1985[224]
Standards/straight chain fatty acid esters of various carotenoids	—	Spheri-5-RP-18, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient MeOH–MeCN–CH_2Cl_2–Hex Flow rate—0.7 mL min^{-1}	PDA 450 nm, except 442 nm for violaxanthin, 400 nm for auroxanthin	—	*J. Chromatogr.*, 449, 119, 1988[225]

Plasma/ 18 carotenoids	Add EtOH, vortex and centrifuge, remove solution. Reextract residue (2×). Dry combined EtOH layers over Na_2SO_4, evaporate, dissolve residue in CH_2Cl_2. Evaporate to dryness, dissolve in mobile phase (isocratic)	Microsorb C_{18} or Silica-based nitrile bonded, 5 μm, 25 cm × 4.6 mm Mobile phase—silica-isocratic Hex:CH_2Cl_2:MeOH:DIEA (74.65:25:0.25:0.1) Flow rate—1 mL min^{-1} MicroSorb—gradient MeCN–MeOH–CH_2Cl_2–Hex Flow rate—0.7 mL min^{-1}	PDA MS w/DCI or ECNI	%CV <5 %Recovery >90	*Anal. Chem.*, 64, 2111, 1992;[226] *Meth. Enzymol.*, 213, 205, 1992[227]
Plasma/α-, β-carotene, β-cryptoxanthin, lycopene lutein	Extract sample 6 × w/Hex, centrifuge following each extraction. Combine Hex fractions evaporate, dissolve in mobile phase	Adsorbsphere C_{18}, 5 μm, 10 cm × 4.6 mm Mobile phase—isocratic MeOH:CH_2Cl_2:MeCN (65:25:10) containing 0.025%BHT, 0.001 M BTP Flow rate—1.5 mL min^{-1}	PDA 450 nm	%CV—2.9–4.3	*J. Chromatogr.*, 614, 43, 1993[228]
Plasma/α-, β-carotene, lycopene	Add echinenone (IS) in EtOH, vortex, add Hex, vortex, centrifuge. Remove 100 μL Hex, evaporate, and dissolve residue in mobile phase	Ultrasphere ODS, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:CH_2Cl_2:MeOH (80:10:10) Flow rate—1.7 mL min^{-1}	464 nm	—	*Am. J. Clin. Nutr.*, 59, 896, 1994[229]
Serum/ β-carotene, 9-*cis*-, 13-*cis*-β-carotene	Add EtOH, vortex, add Hex, vortex, centrifuge. Remove Hex layer, reextract, dry Hex over Na_2SO_4. Clean-up: Sep-Pak alumina	$Ca(OH)_2$, 25 cm × 4.6 mm Mobile phase—isocratic *P*-methylanisole:A:Hex (1:1:98) Flow rate—gradient	410 nm	%Recovery >90	*Clin. Chem.*, 36, 1986, 1990;[230] *J. Agric. Food Chem.*, 42, 2746, 1994[231]
Plasma, blood cells/β-carotene, 9-*cis*-β-carotene	Add EtOH containing 0.015% BHT, shake under N_2. Add 0.9% NaCl, extract w/Hex, evaporate, dissolve residue in EtOH	Vydac C_{18} 201TP54, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:MeCN (95:5) Flow rate—1 mL min^{-1}	450 nm	—	*Lipids*, 30, 493, 1995[232]

Continued

Table 1.12 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Biologicals (*Continued*)					
Serum/α-, β-carotene, lutein, zeaxanthin, β-cryptoxanthin, *cis*-isomers	Add β-apo-10′-carotenal (IS) in EtOH containing 30 μg mL^{-1} BHT, vortex. Add Hex, vortex, centrifuge, reextract, evaporate and dissolve residue in EtOH containing 30 μg mL^{-1} BHT	C_{18} Narrow-bore polymeric, 5 μm, or C_{30}, 3 μm, polymeric, 25 cm × 4.6 mm Mobile phase—gradient MeCN–MeOH–0.05 M NH_4OAC–EtOAC for C_{18} Water–MeOH–0.05 M NH_4OAC–0.05% TEA–MTBE for C_{30}	PDA 250–450 nm	—	*J. Chromatogr*. B, 678, 187, 1996[233]
Serum, human milk/ 34 carotenoids including 13 geometric isomers and 8 metabolites	Serum—extract w/EtOH 1× and Hex 2×, evaporate to dryness, dissolve in eluent B. Human milk—digest sample w/ Pronase E and lipase B. Extract w/ EtOH 1× and Hex 2×, evaporate and dissolve residue in CH_2Cl_2, filter, evaporate under N_2, add eluent B	Microsorb C_{18} or Sillica-based nitrile, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient for C_{18} MeCN–MeOH–CH_2Cl_2–Hex Isocratic for Sillica–Eluent B Hex:CH_2Cl_2:MeOH:DIEA (74.65:25:0.25:0.2) Flow rate—0.7 mL min^{-1}	PDA and LC-MS w/ECNI	%Recovery >95	*Anal. Chem*., 69, 1873, 1997[234]
Plasma, foods/ lycopene, 5-*cis*-, 9-*cis*-, 15-*cis*-	Food—homogenize sample w/$MgSO_4$ and A, filter. Reextract ≥ 2× w/A till extract is colorless. Evaporate combined extracts. Clean-up: aluminum oxide column Plasma—add 0.2 mL water and 0.4 mL EtOH to 0.2 mL clarified plasma, vortex, add 0.8 mL Hex, shake 10 min. Centrifuge, evaporate 0.5 mL Hex layer, add 10 mL CH_2Cl_2 and dilute w/0.25 mL Hex	Nucleosil 300-S, three column in series, 25 cm × 4.6 mm Mobile phase—isocratic Hex containing 015% *m*-ethyladiisopropylamine Flow rate—1 mL min^{-1}	PDA 470 nm	%Recovery 90–94 (food) 95–102 (plasma)	*Food Chem*., 59, 459, 1997[235]
Blood plasma/ carotenoids	Extract sample w/Hex following the protein precipitation w/MeOH. Evaporate the extract and redissolve in MeOH–THF (50:50).	Vydac 218P54, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:THF (90:10) Flow rate—1 mL min^{-1}	Thermal lens detection 476nm/632.8 nm or UV/VIS 450 nm	DL—70–120 pg mL^{-1}	*J. Chromatogr. B*, 718, 47, 1998[236]

Human plasma/ 13 carotenoids	Add EtOH containing IS to deproteinize the sample. Extract the sample w/Hex, 2×. Evaporate the combined extracts to dryness, redissolve residue in MeOH: CH_2Cl_2 (35:65)	Nucleosil C_{18}, 3 µm, 15 cm × 4.6 mm and Vydac C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:MeOH (containing 50 mM NH_4OAC):water:CH_2Cl_2 (70:15:5:10) Flow rate—2 mL min^{-1}	PDA 450 nm	$\%CV_{Intra}$—4.5–8.3 $\%CV_{Inter}$—1.3–12.7	*J. Chromatogr*. B, 751, 297, 2001[237]
Plant, animal tissue, human serum/retinoids, carotenoids	Extract sample using A and Hex w/water	NP: Microsorb, 3 µm, silica, 10 cm × 3.6 mm. Mobile phase—isocratic Hex:IPA:HAC (100:0.5:0.1) Flow rate—1 mL min^{-1} RP: Microsorb, 3 µm, C_{18}, 10 cm × 3.6 mm Mobile phase—gradient MeCN-CH_2Cl_2-water-10 mM NH_4OAC	UV/Vis	%Recovery ≥95	*J. Chromatogr. A*, 936, 71, 2001[238]
Human serum/ 21 carotenoids	Mix sample w/water and 0.01% AA, add A to the mixture, and extract it w/Hex. Evaporate Hex layer to dryness. Redissolve the residue w/MeOH:CH_2Cl_2 (55:45)	YMC C_{30}, 5 µm, 25 cm × 4.6 mm Mobile phase—gradient MeOH–CH_2Cl_2 Flow rate—1.0 mL min^{-1}	PDA 476 nm	—	*J. Chromatogr*. B, 824, 99, 2005[239]
LC-MS					
Serum/lycopene, α-β-carotene	Mix sample w/IS, vortex. Extract sample mixture w/Hex, 2×. Evaporate combined extracts to dryness, redissolve in EtOH	Mightsil C_{18}, 5 µm, 75 × 4.6 mm Mobile phase—isocratic MeOH:MeCN (70:30) Flow rate—1 mL min^{-1}	MS-APCI positive ion mode w/SIM	%Recovery—89–102 %CV—5.3–11.2	*J. Chromatogr*. B, 708, 67, 1998[240]
Chicken plasma/ carotenoids	Mix 1 mL EtOH containing 1% BHT w/0.5–1.0 mL sample, add β-apo-12′-carotenal (IS), stir. Add 2 mL Hex, stir, centrifuge, collect organic phase, repeat extract. Evaporate the combined organic phase, redissolve residue in TBME:MeOH (1:1)	YMC C_{30}, 5 µm, 25 cm × 4.6 mm Mobile phase—gradient TBME–MeOH–water Flow rate—1 mL min^{-1}	MS-APCI positive ion mode	—	*Poultry Sci*., 82, 395, 2002[241]

Continued

Table 1.12 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
LC-MS (*Continued*)					
Protist/carotenoids	Moister the lyophilized cells w/water and extract w/$CHCl_3$:MeOH (3:1), repeat at least 3 × till the cell debris is colorless. Evaporate the extract by vacuum. Redissolve residue in $CHCl_3$ Clean-up: Silica gel column	Shim-pack VP-ODS, 15 cm × 2 mm Mobile phase—gradient MeOH–water Flow rate—0.2 mL min^{-1}	MS-APCI positive ion mode	—	*Biosci. Biotechnol. Biochem.*, 67, 884, 2003[242]
Plasma/carotenoids	Mix plasma with [$^{13}C_6$]-β-carotene (IS). Extract with hexane. Evaporate to dryness, redissolve in MeCN: MTBE (1:1)	Lycopene *cis*- and *trans*-isomers YMC—Pack C_{30}, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient MeOH–MTBE (40:60) Flow rate—0.65 mL min^{-1}	MS-MS-APCI positive and negative ion modes	—	*Anal. Chem.*, 57, 812, 2003[243]
Plasma/carotenoids, retinol	Add β-apo-8′-carotenal and retinol-d8 (IS). Extract with Hex. Dry under N_2 and redissolve in MTBE:MeOH (1:1)	YMC—Pack C_{30}, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient 1 mM ammonium acetate in MeOH–MTBE Flow rate—1 mL min^{-1}	MS-APCI positive ion mode	—	*J. Agric. Food Chem.*, 51, 4877, 2003.[244]

use excessive solvent volumes to achieve satisfactory recovery of the analytes. Saponification parameters are discussed in detail for vitamin E analysis (Chapter 3). Issues relevant to the use of saponification for extraction of vitamin E apply to the extraction of retinol and the carotenoids. Details of several applications of saponification for retinol and carotenoid extraction are given in Table 1.13.

Saponification destroys lipids, chlorophyll, and other materials in the sample that potentially would interfere with chromatographic separations. Partitioning of the analytes into the organic phase can be problematic and the behavior of various partitioning solvents can be difficult to predict with complex sample digests. Following hydrolysis, the digest is diluted with water or salt solution to inhibit emulsion formation, and organic solvent is added to extract the nonsaponifiable fraction. Solvents used include hexane, diethyl ether, 1:1 mixtures of hexane and diethyl ether, and hexane containing up to 15% acetonitrile. Hexane can be used as the extracting solvent, which decreases soap extraction that occurs with diethyl ether.[111] The effect of soaps on the partitioning of the saponified analytes from the aqueous phase to the organic phase must be controlled.

Thompson[111] relates use of hexane to low vitamin recoveries. In the digest, the ethanol-water-soap mixture tends to behave similarly to a hydrocarbon solvent, decreasing the affinity of the fat-soluble vitamins to the organic phase. High ingoing fat levels with increased soap formation can increase the effect with resulting low analyte recoveries. Thompson[111] stressed that the efficiency of extraction with hexane is dependent on the concentrations of fatty acids in the digest. Control is achieved by limiting the amount of fat in the sample (sample weight), by optimizing the amount of water added before extraction, and by repeated extractions with small volumes of hexane. Examination of the methods in Table 1.13 shows that hexane, petroleum ether, diethyl ether, and hexane:diethyl ether mixtures are common extracting solvents. Extended, ambient temperature saponification decreases heat-induced isomerization. Digests must include antioxidants such as ascorbic acid, pyrogallol, and/or BHT. Flushing the vessel with inert gas, usually nitrogen, provides additional protection from oxidation, and protection from light and metal contamination must be assured during all stages of the sample extraction process. To further inhibit oxidation, an antioxidant such as BHT, which is soluble in the organic phase, should be included in the extracting solvent. Temperature affects on stability of analytes needs careful consideration. For some matrices, higher temperatures might increase carotenoid extraction but can result in lower xanthopyll recovery.[122]

1.3.2.2.1.2 Direct solvent extraction. Many organic solvents and solvent mixtures efficiently extract retinoids and carotenoids directly from the sample matrix. Solvent systems must be capable of penetrating tissues and breaking lipoprotein bonds to free the analytes.[112] Under most circumstances, complete lipid extraction must be assured to accomplish efficient removal of retinoids and carotenoids from the sample matrix. Most physiological fluids are extracted with simple direct solvent extraction procedures. These protocols follow the sequence as follows:

1. Denaturing proteins with a volume of ethanol, methanol, or acetonitrile equal to the sample volume
2. Addition of buffer or water to improve the extraction efficiency of the solvent
3. Addition of organic phase to extract the retinoids and carotenoids
4. Centrifugation to facilitate phase separation
5. Solvent evaporation
6. Examination of the methods provided in Tables 1.11 and 1.12 shows that hexane is commonly used as the extracting solvent. Other solvents include acetone, diethyl ether, chloroform, ethyl acetate, acetonitrile, tetrahydrofuran, and various

Table 1.13 Saponification Parameters for Retinol and Carotenoids

Matrix	Sample size	Hydrolysis conditions	Antioxidant	Extractant	Internal standard or % recovery	Quantitation level	References
Retinol							
Dried skim milk	20 g	Ethanolic KOH reflux, 30 min	Sodium ascorbate	Petroleum ether	None	—	*Int. Dairy Fed.*, Bulletin 285, 53, 1993;[152] *J. Food Compos. Anal.*, 6, 3, 1993;[153] *Die Nahr.*, 38, 527, 1994[154]
Milk	200–1000 mg	Ethanolic KOH 70°C, 20 min	Ascorbic acid	Heptane:diisopropyl ether (3:1)	99%	0.5 µg g^{-1}	*J. Dairy Res.*, 61, 233, 1994[155]
Liver, liver products	1–5 g	Ethanolic KOH Ambient, overnight	Ascorbic acid Nitrogen flush	Dilute to water: alcohol ratio of 1:1 Pass through Kieselguhr column Elute retinol with petroleum ether	None	—	*Z. Lebensm. Unters. Forsch.*, 199, 206, 1994[156]
Foods Milk Liver	1–10 g	Ethanolic KOH Ambient, 16 h	BHT Ascorbic acid Nitrogen flush	Dilute to water: ethanol ratio of 1:1 Pass through Kieselguhr column Elute with petroleum ether	None	4.2 mg 100 g^{-1}	*J. Chromatogr.* A, 693, 271, 1995[157]
Retinol, β-carotene							
Human plasma, rat liver	0.15 mL serum 0.3 mL liver homogenate	Ethanolic KOH	Ascorbic acid, BHT	Hexane (0.025% BHT)	%Recovery (β-carotene) Serum—99.7 Liver—93.7 %Recovery (retinol) Serum—107.1 Liver—92.8	—	*J. Chromatogr. B*, 791, 305, 2003[184]

Dairy products	1 mL milk, 1 g cheese	Extract fat with diethyl ether, containing 0.0025% BHT Saponify with KOH, 3 h, ambient	Pyrogallol	Hexane		%Recovery >80	*J. Food Compos. Anal.*, 19, 67, 2006[249]
Carotenoids							
Berries, fruits, vegetables	5–10 g	Extract with acetone Ethanolic KOH Ambient, overnight	Ascorbic acid	Hexane:diethyl ether (70:30)	β-carotene—101% Lycopene—103% Lutein—103%	4.0 μg 100 g^{-1}	*J. Agric. Food Chem.*, 37, 655, 1989[193]
Fruit	50 g	Ethanolic KOH Ambient, overnight	Ascorbic acid Nitrogen flush	Hexane (0.01% BHT)	α-carotene—82% β-carotene—87% $n = 52$	2.0 μg 100 g^{-1}	*J. Food Compos. Anal.*, 3, 119, 1990[194]
Vegetables	10 g	Solvent extraction Methanolic KOH Ambient, 16 h	Nitrogen flush	Hexane	β-apo-8′-carotenal	0.6 μg g^{-1}	*J. Food Prot.*, 56, 51, 1993[245]
Sweet potato	5–15 g	Blanch potato Ethanolic KOH Ambient, overnight	Ascorbic acid Nitrogen flush	Hexane (0.01% BHT)	α-, β-carotene—90%–100% $n = 28$	1.1 μg g^{-1}	*J. Food Compos. Anal.*, 6, 336, 1993[195]
Human milk	4.0 mL	Ethanolic KOH Ambient, 5 h		Hexane			*J. Nutr. Biochem.*, 5, 551, 1994[246]
Fruit Vegetables	10 g	Extract with THF:MeOH (1:1) with magnesium carbonate Partition with petroleum ether Evaporate Redissolve in CH_2Cl_2 Saponify with methanolic KOH Ambient, 1 h	BHT	Petroleum ether	β-Apo-8′-carotenal or echiemone	6 μg 100 g^{-1}	*Food Chem.*, 54, 101, 1995[247]
Dark green vegetables	25 g	Blanch vegetables Extract with acetone:petroleum: (0.5% BHT) (3:2) Saponify with ethanolic KOH Ambient, 15 min	BHT	Water wash to remove acetone	β-carotene—96%–104%	—	*Food Chem.*, 55, 63, 1996[47]

solvent mixtures. Various solvent mixtures detailed by Rodriguez-Bernaldo de Quirós and Costa[122] include acetone:methanol (50:50), tetrahydrofuran: methanol (50:50), hexane:ethyl acetate (85:15), and ethanol:hexane (4:3). Numerous other solvent mixtures are cited in the large number of references available in the literature on extraction of carotenoids from fruits and vegetables. Further extract cleanup by SPE can be completed but is usually not necessary for assay of retinol and its metabolites from physiological fluids. Retinoic acid and the retinoid glucuronides require acidification or addition of buffer salts to the solvent for efficient extraction. Acetonitrile is an excellent solvent for extraction of retinoic acid.[109] All-*trans*-retinoic acid and its *cis*-isomers were extracted from plasma in more recent work by a combined solvent-SPE extraction.[249] Isopropyl alcohol was used to denature the protein, following addition of acetonitrile containing 0.01 M BHT. After centrifugation, 1% ammonium acetate solution was added to reduce the solvent concentration and ensure SPE retention of the lipophilic retinoic acids onto the SPE column (Methyl-C1, Accubond). Retinoids were eluted with acetonitrile/0.01 M BHT.

Under certain conditions, carotenoids can be efficiently extracted from fruit and vegetables by direct solvent extraction. Khachik and coworkers[189–191] used the following procedure to extract carotenol esters for characterization from squash and various fruits:

1. Add tetrahydrofuran, sodium sulfate (200% of the sample weight), and magnesium sulfate (10% of the sample weight)
2. Blend for 5 min with a blender
3. Filter and reextract the residue until the filtrate is colorless
4. Evaporate with rotary evaporator to near dryness at 30°C
5. Partition the concentrated extract between petroleum ether and water (salt can be added to break emulsions)
6. Wash the aqueous layer with petroleum ether containing 15% methanol several times until colorless
7. Combine organic layers and dry over sodium sulfate
8. Evaporate, dissolve residue in hexane

Isozeaxanthin dipelargonate and β-apo-8′-carotenol served as internal standards. The internal standards were added before blending of the sample.

Rodriguez-Bernaldo de Quirós and Costa[122] summarized precautionary measures needed for solvent extraction of carotenoids as follows:

9. Extractions must be carried out rapidly, in the dark, and without exposure to oxygen, high tempertatures, and prooxidant metals
10. Addition of antioxidants to the extraction solvent is essential.
11. Addition of manesium carbonate or calcium carbonate in advisable to neutralize trace organic acids
12. Tetrahydrofuran and ethyl ether can rapidly develop peroxides that will enhance oxidation of the carotenoids. Addition of antioxidants to the solvents will increase stability.

1.3.2.2.1.3 Supercritical fluid extraction. Turner and colleagues[250] state that the critical point of a fluid (C_p) is defined by its critical pressure (P_c) and temperature (T_c). Only one state, the supercritical fluid state, exists at temperatures above the critical point. The supercritical fluid has a uniform density with no distinction between the gaseous and liquid phases. With increasing pressure, the density approaches that of a liquid solvent with properties similar to a liquid. Supercritical fluids also have gas-like viscosities that enhance penetration into sample matrices.

Supercritical fluid extraction (SFE) is an extraction technique, which exploits the solvent properties of fluids above their critical point. Carbon dioxide is often used instead of the organic solvent that is normally employed in conventional extraction methods. The extraction conditions are related to the relative solvent strength, which, in a given fluid, is primarily dependent upon its density. The selectivity of the extraction can be optimized by adjusting the conditions of temperature and pressure; thus, it reduced the need for a complex cleanup stage before analysis.[250] SFE eliminates harmful organic solvents in the extraction of lipophilic plant components and avoids environmental pollution. Marsili and Callahan[251] compared a liquid solvent extraction technique with SFE for the determination of α- and β-carotene in vegetables. They concluded that SFE provides recoveries of α- and β-carotene from vegetables that are equal to or greater than recoveries obtained with traditional solvent extraction techniques.

Turner and colleagues developed an online SFE/enzymatic hydrolysis procedure using immobilized lipase for the determination of vitamin A and E in food.[252] Immobilized lipase and 0.5 g sample (mixed with 1 g of Hydromatrix) were loaded into the extraction cell, separated by a small layer of Hydromatrix. Two milliliters of ethanol containing 5% water (v/v) and 0.1% BHT (w/v) was then added on top of the sample mixture to facilitate better extraction. Water was added to speed the enzymatic reaction. Lastly, the remaining cell volume was filled with Hydromatrix. The samples were extracted with supercritical carbon dioxide modified with 5% (v/v) ethanol and collected into 10 mL of ethanol containing 0.1% BHT (w/v). The extract was evaporated to dryness under nitrogen gas and redissolved in 1 mL of ethanol. The recoveries of vitamin A were over 90%. This method is faster and more automated than SFE with offline saponification or conventional extraction techniques because no additional saponification or cleanup steps are needed. SFE has been applied to lycopene extraction from tomato products,[253–255] and carotenoids from carrot.[256,257]

1.3.2.2.2 Chromatography parameters

1.3.2.2.2.1 Supports and mobile phases. The majority of LC methods for resolution of retinoids and carotenoids rely on reversed-phase C_8, C_{18}, or C_{30} supports (Tables 1.11 and 1.12). Advantages of reversed-phase systems compared to normal-phase chromatography include the following:

1. Less sensitive to changes in retention time owing to the presence of water
2. More easily cleaned of contaminants
3. More stable to small changes in mobile phase composition
4. More quickly equilibrated to mobile phase composition changes, permitting use of gradients
5. Capable of resolving compounds with a wide range of polarities[34,106,107,109,117]

Both isocratic and gradient mobile phase systems are provided in the methods summarized in Tables 1.11 and 1.12. For retinol analysis by reversed-phase chromatography, simple, isocratic, methanol–water, acetonitrile–water, or gradients based on these solvents provide excellent resolution. When more polar metabolites (retinoic acid) or synthetic retinoids are under study, acetonitrile- or methanol-based mobile phases are modified by the addition of 1% or 0.1 M ammonium acetate or acetic acid. Acid addition results in ion suppression of the carboxylic acids but does not affect resolution of retinol or retinyl esters. Ammonium acetate improves resolution and decreases broadening of the retinoic acid peak.[107]

Normal-phase or absorption chromatography is advised when critical resolution of retinol isomers is required. Hexane or heptane mobile phases modified with isopropanol, 1,4-dioxane, or *tert*-butyl methyl ether resolve retinol isomers more efficiently than reversed-phase systems. Retinoic acid chromatography on silica support requires acidification of the mobile phase, usually with acetic acid, to decrease the affinity of the polar analyte for

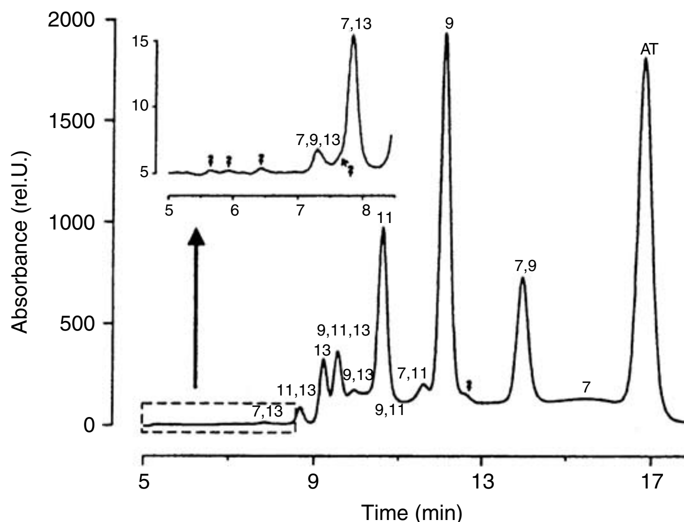

Figure 1.7 Chromatograms of retinal isomers on Zorbax SIL. Mobile phase was hexane:*tert*-butyl methyl ether (97:3), 2 mL min^{-1}. (Reproduced with permission from Nöll, G. N., *J. Chromatogr. A*, 721, 247, 1996.)

the support. Wyss[107] reviewed mobile phase composition and applications of various chromatography systems to retinoid resolution.

Nöll[258] and Nöll and Kalinowski[259] showed that identification of retinol isomers can be easily misidentified and that some identifications in the literature are inconsistent or wrong. Using Zorbax SIL and LiChrospher Si60 supports with *n*-hexane:5-butyl methyl ether (97:3) mobile phase, retinol and retinal isomers were detected at 325 nm and 371 nm, respectively. A characteristic chromatogram is shown in Figure 1.7. Nöll[258] stressed that elution time and pressure of the system are dependent on the columns used. Factors such as specific inner surface area of the support can affect resolution time and order of elution of the various isomers. Nöll and Kalinowski[259] compared many previous chromatographic systems (51 papers) and showed that inconsistencies exist in identification of eluted peaks. They again stressed that the high performance liquid chromatography (HPLC) mode, support material, mobile phases, ratio of eluents, flow rate, pressure, detection wavelength, and column temperature are all important factors controlling retention time and order of elution of complex retinoid isomeric mixtures.

O'Neil and Schwartz[120] reviewed the chromatography of *cis*- and *trans*-carotenoid isomers through 1992. For β-carotene isomers, most procedures used $Ca(OH)_2$ or Vydac C_{18} supports. Vydac supports are produced using trichlorosilanes in a polymeric synthesis of C_{18} as opposed to monomeric synthesis with monochlorosilanes. The polymeric C_{18} has greater shape selectivity toward geometric isomers compared to monomeric supports.[48] Normal-phase chromatography on $Ca(OH)_2$ provides excellent *cis*-resolution from *trans*-isomers; however, the support is not commercially available and in-house packing is irreproducible.[48,120] Therefore, polymeric C_{18} supports have routinely been used for studies on *cis*– and *trans*-carotenoids. Monomeric C_{18} supports provide resolution of some of the xanthophylls.[120] A variety of solvent mixtures including methanol, acetonitrile, dichloromethane, and tetrahydrofuran are useful for carotenoid resolution by reversed-phase chromatography (Table 1.11).

Since C_{18} supports have limited capacity to resolve *cis*- and *trans*-isomers of the carotenoids,[119] a C_{30} stationary phase was designed at the National Institute of Standards and Technology (NIST) to provide high absolute retention, enhanced shape-recognition of

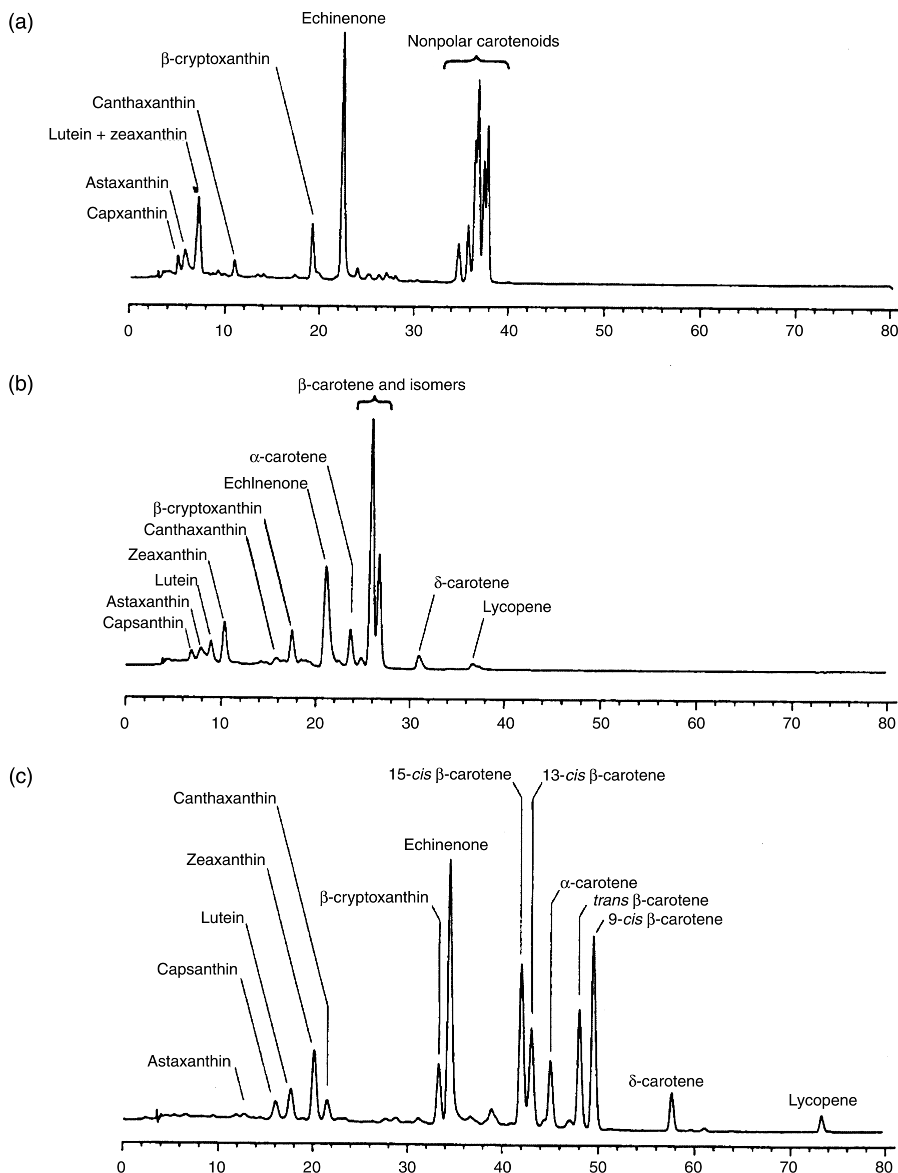

Figure 1.8 Separation of carotenoid standards on commercial (a) monomeric and (b) polymeric C_{18} columns, as well as the engineered C_{30} "carotenoid column" (c). Separation conditions were as follows: 81:15:4 to 6:90:4 methanol/methyl-*tert*-butyl ether/water of 90 min; 1 mL min^{-1}; 20°C. (Reproduced with permission from Sander, L. C., Sharpless, K. E., Craft, N. E., and Wise, S. A., *Anal. Chem.*, 66, 1667, 1994.)

carotenoid isomers, and moderate silanol activity.[105] The support was produced by triacontyl (C30) polymeric surface modification of a moderate pore size (~20 nm), moderate surface area (~200 m^2 g^{-1}) silica, without endcapping. Superior resolution was obtained compared to monomeric and polymeric C_{18} supports. Figure 1.8 compares resolution of carotenoids and *cis*- and *trans*-isomers on the monomeric, polymeric C_{18}, and C_{30} carotenoid column.[105] The C_{30} support has rapidly become a major tool for the resolution of complex carotenoid profiles.[48,49,130,183,202,204,207,213,217–219,221,223,239,241,243,244,247,256,260,261,263,264]

In addition, NIST synthesized and characterized a new long-chain (C_{34}) alkyl-bonded support for carotenoid resolution.[265] This stationary phase was synthesized by polymeric and surface-polymerization synthesis. The C_{34} column slightly improved carotenoid isomer separation compared to the C_{30} stationary phase. The improvement was associated with the ability of large carotenoid molecules to more fully interact with the thicker C_{34} support. The ability to engineer 'designer' supports like the C_{30} and C_{34} polymeric supports that incorporate shape selectivity or other properties of a specific analyte or class of analytes add a significant dimension to LC capabilities.

An interlaboratory study completed in Europe gives important data about the capability of several different supports and mobile phases used to determine β-carotene in commercial foods.[266] The interlaboratory study included 14 different laboratories assaying four products with saponification and direct solvent extraction. Liquid chromatography conditions were the laboratories' normal operating systems. All methods resolved lycopene, α-carotene, and β-carotene. Repeatability (RSD_r) for total β-carotene was quite good (2.9%–5.6%). Reproducibility (RSD_R) ranged from 6.5% to 15%. Values for all-*cis*-β-carotene were 3.3%–5.1% (RSD_r) and 8.4%–14% (RSD_R). The following major conclusions were drawn from the study:

1. Exclusion of *cis*-isomers of β-carotene can underestimate the effective β-carotene content.
2. Direct solvent extraction can be used for most samples if the method verifies the absence of interfering compounds by saponifying the sample after direct extraction and comparing the chromatograms. Because of the extent of the information provided by the study, the supports and mobile phases and their capabilities are reproduced from the paper in Table 1.14. However, if accurate determination of vitamin A activity is an objective of the assay, resolution of *cis*- from all-*trans*-β-carotene is required.

1.3.2.2.2.2 Detection

Retinoids: The conjugated double-bond system present in natural and some synthetic retinoids gives quite specific and strong UV absorbance properties (Section 1.2.1.2). Most LC procedures that use UV absorbance provide quantitation limits less than 1 ng mL^{-1}. λ_{max} are usually between 320 and 380 nm (Table 1.3). Fluorescence can be used for retinol and retinyl esters (Exλ = 325, Emλ = 470); however, oxidation of the alcohol essentially destroys the fluorescence, and retinoic acid and many synthetic retinoids cannot be detected by fluorescence. Retinol, bound to the retinol-binding proteins, fluorescences at Ex λ = 333, Em λ = 470.[43] Electrochemical detection has been used in a few studies. Hagen and colleagues[267] applied capillary LC and amperometric detection to improve detection limits three orders of magnitude compared to UV detection. Detection limits were between 0.267 and 2.73 fmol for the retinoids included in the study. The methodology was developed for assay of extremely limited quantities of embryonic tissue. Although the use of electrochemical detection is advantageous in cases where sample amounts are highly limited, most routine assays do not require such low detection limits, and UV detection is more than sufficient. PDA detectors with increased sensitivity, compared to detectors introduced throughout the 1980s, are used extensively for multiple vitamin assays. Such methods are covered in Chapter 14.

Carotenoids: The carotenoids show strong absorption bands in the visible region owing to the long conjugated double-bond system.[40] Maximum absorbance is usually between 400 and 500 nm, but the λ_{max} of individual carotenoids can vary. The strong, characteristic, visible absorption provides an ideal detection mode; however, 450 nm, a wavelength near the λ_{max} of β-carotene, is not the best for detection of many other carotenoids (Tables 1.4 and 1.5).

Table 1.14 LC Conditions Used by Participants in European Interlaboratory Study on Carotenoid Analysis[266]

Laboratory	Precolumn	Stationary phase	Mobile phase	Flow mL min^{-1}	Column temp, °C	Detection wavelength, nm	Retention times, min			
							Lycopene	α-Carotene	(all-E)-β-Carotene	(Z)-β-Carotene
1	None	Spherisorb ODS-2, 5 µm, 250 × 4.0 nm	CH_3CH-0.05 *M* $AcONH_4I$ in CH_3OH-CH_2Cl_2 (75 + 20 + 5)	1.0	RT[a]	450	10.5	17.9	18.8	19.8
2	Spherisorb ODS-2, 10 × 4.6 mm + 100 × 4.6 mm	Bydac 201 TD, 5 mm, 250 × 4.6 mm	CH_3CN-CH_3OH-CH_2Cl_2 (75 + 20 + 5)	1.5	22.5	450	16.0	20.4	22.8	2.2
3	None	Nucoeosil 100-10 C_{18}, 10 µm, 250 × 4.0 mm	CH_3OH-CH_3CN-2-propanol-0.2% $AcONH_4$[b] in H_2O-*N*-ethyldiisopropylamine (500 + 544 + 20 + 25 + 0.2) + 50 ppm BHT[c]	1.0	30	445	18.6	25.7	28.0	29–32.1
4	Suplex pKb-100, 5 µm, 30 × 4.6 mm	Suplex pKb-100, 5 µm, 250 × 4.6 mm	CH_3OH-CH_3CN-2-propanol-0.2% $AcONH_4$ in H_2O-*N*-ethyldiisopropylamine (500 + 455 + 25 + 0.2) + 50 ppm BHT	1.0	30	445	16.0	21.3	22.9	24.4–27.5
5	RP18, 10 × 4.6 mm	Brownlee Sphen-5 ODS, 5 µm	CH_3-CN-CH_3OH-THF[d] (55 + 35 + 15)	0.8	RT		12.3	17.9	19.9	20.7
6	Suplex pKb-100, 5 µm, 30 × 4.6 mm	Suplex pKb-100, 5 µm, 250 × 4.6 mm	CH_3OH-CH_3CN-2-propanol-0.2% $AcONH_4$ in H_2O-N-ethyldiisopropylamine (500 + 455 + 25 + 0.2) + 50 ppm BHT	1.0	21	445	17.8	24.1	26.2	—
7	Lichrospher 100 RP18, 5 µm, 4 × 4.0 mm	Lichrospher 100 RP18, 5 µm, 250 × 4.0 mm	CH_3OH-CH_3CN-2-propanol-0.2% $AcONH_4$ in H_2O (500 + 455 + 25 + 0.2) + 50 ppm BHT	2.2	25	453	31.7	45.5	50.2	53.2
8	None	Nucleosil-100 C_{18} AB, 5 µm, 250 × 4.0 mm	CH_3OH-CH_3CN-2-propanol-0.2% $AcONH_4$ in H_2O (500 + 455 + 25 + 0.2) + 50 ppm BHT	1.5	20	455	14	15	17	18–19

Continued

Table 1.14 (Continued)

Laboratory	Precolumn	Stationary phase	Mobile phase	Flow mL min^{-1}	Column temp, °C	Detection wavelength, nm	Retention times, min			
							Lycopene	α-Carotene	(all-E)-β-Carotene	(Z)-β-Carotene
9	None	Lichrospher 100 RP18, 5 μm, 250 × 4.0 mm	CH_3CN-CH_3OH-THF (40 + 55 + 5)	2.0	30	450	9.2	13.1	14.1	14.6–15.2
10	Lichrospher 100 RP18, 5 μm, 4 × 4.0 mm	YMC, C_{30} polymeric nonendcapped, 5 μm, 250 × 4.6 mm	CH_3OH-MTBE[e] gradient (30-80% MTBE) + 0.1% BHT	1.0	22	452	18.4	11.3	12.4	10.8; 13.4
11	None	Vydac 201 TP54 protein & peptide C_{18}, 5 μm, 250 × 4 mm	CH_3OH-0.05 *M* $AcNH_4$ in CH_3OH-CH_2Cl_2 (75 + 20 + 5)	1.0	15	445	20.3	19.6	22.3	24–28
12	None	Vydac 201 TP54 protein & peptide C_{18}, 5 μm, 250 × 4.6 mm	CH_3OH-THF (99 + 1)	0.6	RT	450	22.8	17.2	19.6	21.5–23.5
13	None	Ultrasphere ODS, 5 μm, 250 × 4.6 mm	CH_3OH-THF (95 + 5)	3.0	RT	450	12	13.6	14.6	—
14	Vydac 201 TP54, 10 × 4.6 mm	Vydac 201 TP54, 10 μm, 250 × 4.6 mm	CH_3OH-THF (95 + 5) + 0.1% BHT	1.0	20	445	24.8	13.5	15.6	17.6

[a] RT, room temperature.
[b] $AcONH_4$, ammonium acetate.
[c] BHT, butylated hydroxytoluene.
[d] THF, tetrahydrofuran.
[e] MTBE, methyl *tert*-butyl ether.

Source: Reproduced with permission from, Schüep, W. and Schierle, J., *J. AOAC Int.*, 80, 1997, 1057.

Variable wavelength, programmable UV/visible and PDA detectors are powerful research tools for study of complex, natural carotenoid mixtures. A multichannel PDA detector is essential for carotenoid research. Peak identification and validation of homogeneity are simplified by capabilities of the modern PDA detector. Further, multichannel capability allows detection of carotenoids with absorption maxima in the low visible to UV range (phytoene, λ_{max} 285; phytofluene, λ_{max} 347;[34] γ-carotene, λ_{max} 437; and lutein, λ_{max} 421).

Mass Spectrometry: Since LC mass spectrometric (MS) coupled instruments became more common in this past decade, the application of MS to structurally identify and quantify retinoids and carotenoids has dramatically increased. The interfacing of LC and MS represents a major advance for the quantification and structural conformation of each of the fat-soluble vitamins, but particularly for the carotenoids, gas chromatography–mass spectrometric (GC-MS) cannot be used for analysis of the carotenoids because of their instability at temperatures required for GC resolution.

van Breemen and Huang[181] and van Breemen[268] traced the evolution of LC-MS for carotenoid analysis, showing technological advances in methodology with improvement in analytical results. In general chronological order, particle beam, fast atom bombardment, electrospray ionization, and atmospheric pressure chemical ionization (APCI) have been successfully applied. Development of electrospray and ACPI techniques substantially improved quantification at analyte levels present in LC eluants of biological samples. Solvent removal and ionization take place at atmospheric pressure and solvent splitting is not required.[268] Coupling LC-MS with the resolution power of the C_{30} reversed-phase support for carotenoids greatly increased the ability to resolve and unambiguously identify components of complex carotenoid mixtures often encountered in food and other biological samples. Details of several LC-MS methods representing various MS techniques are given in Tables 1.11 and Table 1.12.[161,180–183,217–223,240–244] Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is used;[161,221,243] but, the most common technique is LC-MS with positive ion APCI or electrospray in the positive ion mode.[161,180–183,217–219,222,240–242]

1.3.2.2.2.3 Internal standards. Availability of synthetic retinoids simplified the search for useful internal standards for retinol, retinoic acid, and their metabolites. Etretin,[107,168,169] RO11-6738,[107,168,169] RO13-6307,[172] acitretin,[107,180] and 11-*cis*-acitretin[107,180,269] have been used as internal standards in the assay of retinoic acid and its metabolites. 13-*cis*-retinoic acid is a good internal standard in the assay of synthetic retinoids.[168] If saponification is not used in extraction, retinyl esters not present in the sample can serve as the internal standard for retinol and retinyl esters.[173,196] Lanvers and colleagues[269] used arotinoid ethyl sulfone as the internal standard for assay of all-*trans*-retinol and all-*trans*-retinoic acid, various *cis*-retinoic acid isomers, and their 4-oxo metabolites.

β-Apo-8′-carotenal is the most common internal standard for carotenoid assay.[185,186,189,190,197,199,233,244,246] Other carotenoid internal standards include *trans*-β-apo-10′-carotenal oxime,[233,260,270,271] decapreno-β-carotene,[185] monoapreno-β-carotene,[188] echinenone,[224,229,246,272] nonapreno-β-carotene,[188] and isozeaxanthin.[189,190] $^{13}C_6$-β-carotene,[243] squaline,[240] β-apo-12′-carotenal,[241] β-apo-8′-carotenal,[244] and retinol-d8[244] have been used in LC-MS studies. Internal standards for multivitamin assays are provided in Chapter 14.

1.3.3 *Method applications*

1.3.3.1 *Vitamin A and other retinoids*

1.3.3.1.1 AOAC international methods AOAC International[126] Official Methods for vitamin A assay by LC are available for milk and milk-based infant formula (Table 1.9). The Carr–Price method (Method 974.2) is discussed in Section 1.3.1. Method 992.04

(50.1.02) "Vitamin A (Retinol Isomers) in Milk and Milk-Based Infant Formula, Liquid Chromatographic Method" and Method 992.06 (50.1.03) "Vitamin A (Retinol) in Milk-Based Infant Formula Liquid Chromatographic Method" were collaborated in 1993.[127] Method 992.04 quantitates all-*trans*-retinol and 13-*cis*-retinol after sample digestion at ambient temperature for 15 h in ethanolic KOH and extraction of the digest with diethyl ether: hexane (15:85). Normal-phase chromatography on silica (Apex, 3 mm) is completes with heptane:isopropanol at concentrations that elute 13-*cis*-retinol at 4.5 min and all-*trans*-retinol at 5.5 min. Detection is at 340 nm.

Method 992.06 is specific for milk-based infant formula containing 500 IU or more of vitamin A per reconstituted quart. This method incorporates saponification at 70°C for 25 min as opposed to the 18 h digestion at ambient temperature for Method 992.04. Normal-phase chromatography uses a silica-based cyano stationary support (Sepralyte CN, 5 mm) and hexane:isopropyl alcohol (100:0.25 v/v). Detection is at 336 nm. Use of 336 nm assumes that the relative molar absorptivities of both isomers are virtually equal at 336 nm. IUs are corrected for the lower biological activity of 13-*cis*-retinol palmitate in the sample by a correction factor of 0.75 for the *cis*-isomer. The assumption is made that the *cis*-isomer measured as 13-*cis*-retinol after saponification is not an artifact of the digestion and extraction. A deficiency of the method is failure to adequately follow hydrolysis of all-*trans*-retinyl palmitate and extraction efficiency from the sample digest. Retinyl palmitate standards are saponified with water in place of sample. Recoveries from the standard digest may well vary owing to matrix effects from the sample digest.

Method 992.06 makes no provisions for use of spike recoveries or for incorporation of internal standard methodology. Methods 992.04 and 992.06 provide poor directions for saponification. Digestions are completed in a volumetric flask (992.04) or capped tubes (992.06) without evacuation or inert gas flush. The methods would be improved by use of an air condenser (Chapter 3), air removal, or nitrogen flushing.

AOAC Official Method 2002.06 (45.1.01A) "Retinyl Palmitate (Vitamin A) in Fortified Fluid Milk" was collaborated in 2003.[160] The LC method was developed at the Tennessee Department of Agriculture to provide a rapid and simple assay of retinyl palmitate in fortified milk. Using retinyl acetate as internal standard, milk is extracted with hexane, and the hexane layer is directly used for LC analysis. Chromatography is completed with a normal phase silica column, a mobile phase of hexane:isopropanol (98.85:0.15) and UV detection at 325 nm. Repeatability standard deviations (RSD_r) ranged from 1.5% to 5.7%, and reproducibility standard deviations (RSD_r) ranged from 5.0% to 22.7%. The method does not resolve *cis*- from all-*trans*-reinyl palmitate.

The International Dairy Federation (IDF) provides a method for the LC determination of vitamin A in dried skim milk.[152] The IDF procedure is based on saponification and extraction with light petroleum with reversed-phase resolution on C_8 or C_{18}. The saponification procedure is detailed to provide control of vitamin A degradation during the extraction phase of the method; however, like AOAC International methods,[126] no allowance is made for use of spiked recoveries or internal standards to ensure method conformance for extraction of the retinyl ester used for the standard, retinyl ester, all-*trans*-retinol, or *cis*-retinol isomers in the sample. A detailed summary of the IDF method is provided in Section 1.4.

1.3.3.1.2 European Committee for Standardization Methods The European Committee for Standardization approved an LC method for assay of all-*trans*-retinol and 13-*cis*-retinol in 2000.[273] The method, EN 12823-1, uses saponification, extraction with diethyl ether or other appropriate solvents, and normal phase LC on Si60 with UV or fluorescence detection. The method is presented in a precise and understandable format, providing an excellent guide to all phases of the analysis. $\%RSD_r$ and $\%RSD_R$ values for interlaboratory assays of

margarine (CRM 122) and milk powder (CRM 421) were quite good and defined in an earlier publication.[275]

Comprehensive reviews cited in Section 1.3 are available for methods for all-*trans*-retinol, its esters and isomers, and other retinoids. Several recently published methods are summarized in Table 1.11.

1.3.3.2 Carotenoids

Liquid chromatography is necessary to resolve complex mixtures of carotenoids from plant materials. In addition, a PDA detector is essential for peak identity and homogeneity confirmation.[46] Nevertheless, many laboratories around the world do not have this quite sophisticated instrumentation capability and must rely on older procedures, particularly to quantitate provitamin A activity of foods and diets. Because of this, much interest exists in the application and improvement of such methods.

Provitamin A methods were reviewed by Rodriguez-Amaya[114,115] and Rodriguez-Bernaldo de Quirós[122] with emphasis on the associated problems of relevant methods. Evaluated methods include:

1. AOAC International methods of which Method 941.15 (45.1.03) "Carotene in Fresh Plant Materials" is most pertinent to provitamin A estimation in foods
2. European Cooperation in Scientific and Technological Research in methods (COST)
3. Published open-column methods
4. HPLC methods

AOAC[126] and COST[135] provide excellent procedural guides; however, these procedures are adequate only if β-carotene is the sole contributor of vitamin A activity.[114] In AOAC Official Method 941.15, the carotene fraction is extracted with acetone and hexane and isolated by MgO (activated):Hyflo Super Cel (diatomaceous earth) open-column chromatography. The carotenes pass through the column in visible bands with elution with acetone:hexane (1:9). The entire column eluate is collected, diluted to 100 mL, and absorbance read at 436 nm. Xanthophylls, carotene oxidation products, and chlorophylls remain on the column. Results are reported as mg β-carotene or IU of vitamin A activity. Overestimation of vitamin A activity is common if less active provitamin A carotenoids, other than β-carotene, are present in the sample. Method 941.15 was modified to isolate specific carotenoid fractions for improved accuracy, but such modifications add to the tediousness of the assay.[276]

COST procedures[135] apply to complex foods, total carotenes in fruit, vegetables, unaltered plant materials, and beverages. For assay of complex foods, the sample is saponified (30 min, 60°C) with methanolic KOH. Carotenes are partitioned into diethyl ether, evaporated, and redissolved in hexane. Other matrices are treated with slight modification. For each procedure, water is removed from the hexane fraction by adding ethanol and reevaporating the solvent. Residues are dissolved in hexane and passed through a deactivated alumina column. Carotenes are eluted with hexane, and absorbance is measured at 450 nm. β-Carotene is calculated using a $E^{1\%}_{1cm}$ value of 2590 (hexane). The methods assume that contributions of α-carotene, β-cryptoxanthin, and other more uncommon provitamin A carotenoids are negligible. Rodriguez–Amaya[114] provides a complete review of open-column methods and their problems for vitamin A activity measurement of complex matrices.

Characteristics of HPLC procedures suitable for measurement of provitamin A measurement include

1. Capability to efficiently remove interfering compounds before LC through proper extraction techniques
2. Capability to resolve each carotenoid in the sample extract with provitamin A activity

3. Capability to resolve *cis*- from *trans*-isomers
4. Capability to prove peak identity and homogeneity[113]

Early work on resolution of *cis*- and *trans*-isomers showed that $Ca(OH)_2$ was an effective support when acetone:hexane gradients were applied. However, owing to lack of a commercial source and problems with column-to-column repeatability, $Ca(OH)_2$ was never widely applied to carotenoid research.[120] More recent development of the C_{30} polymeric LC columns, as discussed in Section 1.3.2.2.2.1, enhances capability of LC methodology to accurately measure provitamin A activity.

Excellent studies exist in which HPLC methods were used to quantitate primary provitamin A carotenoids in fruits and vegetables. Some of the papers include Chen et al.[245] (Taiwanese vegetables), Homnava et al.[194,277] (fruits), Simonne et al.[195] (sweet potatoes), van Dokkum et al.[278] (Netherlands diets), Heinonen et al.[193] (Finnish foods), Granado et al.[196] (Spanish vegetables), Biacs and Daood[279] (fruits and vegetables), Minguez–Mosquera and Hornero–Méndez[197] (red peppers), Tonucci et al.[199] (processed tomato products), Chen et al.[87,280] (carrots), Nyambaka and Ryley[47] (dark green vegetables), Granelli and Helmersson[281] (milk), Hart and Scott[247] (fruits and vegetables), Wills and Rangga[282] (Chinese vegetables), Mercadante et al.[200] (mango), Riso and Porrini[272] (vegetables), Konings and Roomans[201] (fruits and vegetables), Pupin et al.[203] (orange juice), Marx et al.[204] (carrot juice), Kimura and Rodriguez–Amaya[205] (leafy vegetables), de Sá and Rodriguez–Amaya[206] (cooked green vegetables), Lin and Chen[202] (tomato juice), Surles et al.[210] (carrots), Cortés et al.[212] (juices), Lakshminarayana et al.[214] (Indian leafy vegetables), Azevedo–Meleiro and Rodriguez-Amaya[220] (tropical fruits), and Schweiggert et al.[221] (red peppers). The Konings and Roomans procedure[201] provides an easy to follow protocol and good method performance validation parameters. The procedure is presented in Section 1.4.

Concentrated efforts have extensively characterized the carotenoid profiles of specific fruits and vegetables. Khachik and coworkers at the USDA, Beltsville, Nutrient Composition Laboratory developed HPLC procedures for resolution of up to 30 components in fruit and vegetable extracts.[119,186,188–192] Components identified included xanthophylls, chlorophylls, hydrocarbon carotenoids, β-carotene *cis*-isomers, and carotenol fatty acid esters. Carotenoids were resolved by several different LC systems, and the isolated carotenoids were identified and characterized by thin layer chromatography, Nuclear magnetic resonance (NMR), mass spectrometry, and diode array UV/visible spectroscopy. The study is described in detail in Reference 119. Mercadente and colleagues[200] characterized the carotenoids in mango. Principle carotenoids identified by mass spectrometry after LC purification were all-*trans*-violaxanthin, all-*trans*-β-carotene, and a *cis*-violaxanthin.

Interest in the antioxidant capability of common dietary carotenoids led to extensive research on profiling plasma and tissue carotenoid levels in order to define diet-health relationships. Definitive studies were not completed until the early 1990s. However, earlier work by Nelis and De Leenheer[283] provided the basis for carotenoid assay of serum. This work identified lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene, and β-carotene as the primary constituents of human serum. Subsequent work by Bieri and colleagues[224] identified an additional, unidentified peak in normal plasma. These authors essentially used the Nelis and De Leenheer method, substituting a C_{18} Supelco column for the Zorbax ODS support used by Nelis and De Leenheer. Comprehensive characterization studies have been completed by research led by Khachik and Beecher at the USDA Nutrient Composition Laboratory that provides extensive information on carotenoids in plasma, human milk, and other physiological samples. Initial studies published in 1991[284] identified cryptoxanthin as the unknown carotenoid found by Bieri and colleagues[224] in earlier work.

Subsequent work identified 18 carotenoids in extracts of human plasma.[226,227] Methods available for plasma analysis up to 1992 were reviewed by Khachik and colleagues[227] Khachik and colleagues[234] identified 34 carotenoids in breast milk and human serum.

Identifications included 13 geometric isomers and 8 metabolites. The procedure included resolution by reversed-phase and normal-phase chromatography, PDA detection, and HPLC-MS interfaced identification by electron capture negative ionization. Because of the significance of this work, the carotenoids identified in this comprehensive study are listed in Table 1.15 along with absorption maxima and molecular mass information. Methodology is summarized in Table 1.12.

AOAC International recently collaborated AOAC Official Method 2005.07 "β-Carotene in Supplements and Raw Materials—Reversed-Phase High-Pressure Liquid Chromatographic Method." The procedure, based upon original work by Schierle and colleagues,[223] carefully details sample preparation steps for oily solutions or suspensions

Table 1.15 LC Peak Identification of Carotenoids in Human Serum and Milk Extracts

Peak no.	Serum/milk carotenoids[a]	Absorption maxima (nm)[c]	Molecular mass (m/z)[d]
Eluent B			
1[a]	ε,ε-carotene-3,3′-dione	420, 442, 472	564
2[a]	3′-hydroxy-ε,ε-carotene-3-one	422, 442, 472	566, 548 (M-H_2O)
3[a]	2,6-cyclolycopene-1,5-diol I	434, 458, 492	570
4[a]	3-hydroxy-β,ε-carotene-3′-one	(424), 448, 476	566, 548 (M-H_2O)
5[a]	(*Z*)-3-hydroxy-β,ε-carotene-3′-one	(420), 442, 470	566, 548 (M-H_2O)
6	(3*S*,6*S*,3′*S*,6′*S*)-ε,ε-carotene-3,3′-diol (lactucaxanthin)	416, 442, 470	566, 550 (M-H_2O)
7	(13*Z*,13′*Z*,3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol [(13*Z*,13′*Z*,3*R*,3′*R*,6′*R*)-lutein]	274, 336, 410, 432, 460	568, 550 (M-H_2O)
8	(all-*E*,3*R*,3′*R*)-β,ε-carotene-3,3′-diol [(all-*E*,3*R*,3′*R*,6′*R*)-lutein]	(424), 448, 476	568, 550 (M-H_2O)
9[a]	2,6-cyclolycopene-1,5-diol II	432, 458, 490	570
10	(all-*E*,3*R*,3′*R*)-β,β-carotene-3,3′-diol [(all-*E*,3*R*,3′*R*)-zeaxanthin]	(428), 454, 482	568
11[a]	(all-*E*,3*R*,3′*S*,6′*R*)-β,ε-carotene-3,3′-diol [(all-*E*)-3′-epilutein]	(424), 448, 476	568, 550 (M-H_2O)
12	(9*Z*,3*R*,3′*R*,6′*R*)-lutein	334, (420), 442, 470	568, 550 (M-H_2O)
13	(9′*Z*,3*R*,3′*R*,6′*R*)-lutein	332, (420), 444, 472	568, 550 (M-H_2O)
14	(13*Z*)-lutein + (13′*Z*)-lutein	334, (418), 440, 468	568, 550 (M-H_2O)
15	(9*Z*)-zeaxanthin	340, (424), 450, 474	568
16	(13*Z*)-zeaxanthin	338, (419), 446, 472	568
17	(15*Z*)-zeaxanthin	338, (426), 450, 478	568
Eluent A			
18[a]	(3*R*,6′*R*)-3-hydroxy-3′,4′-didehydro-β,γ-carotene	334, (424), 446, 476	550
19[a]	(3*R*,6′*R*)-3-hydroxy-2′,3′-didehydro-β-ε-carotene (2′,3′-hydrolutein)	336, (424), 448, 476	550
20	β,ε-caroten-3-ol (α-cryptoxanthin)	(424), 446, 476	552
21	3-hydroxy-β-carotene (β-cryptoxanthin)	(428), 454, 480	552
22	(*Z*)-3-hydroxy-β-carotene [(*Z*)-β-cryptoxanthin]	(424), 450, 476	552
23	ψ,ψ-carotene (lycopene)	446, 474, 502	536
24	(*Z*)-ψ,ψ-carotene [(*Z*)-lycopene]	348, 362, 438, 466, 494	536
25	7,8-dihydro-ψ,ψ-carotene (neurosporene)	418, 442, 470	538
26	β,ψ-carotene (γ-carotene)	(440), 462, 492	536
27	7,8,7′,8′-tetrahydro-ψ,ψ-carotene (ζ-carotene)	378, 400-402, 426	540
28	β,ε-carotene (α-carotene)	(428), 446-448, 474	536

Continued

Table 1.15 (Continued)

Peak no.	Serum/milk carotenoids[a]	Absorption maxima (nm)[c]	Molecular mass (m/z)[d]
Eluent A (*Continued*)			
29	(all-*E*)-β,β-carotene [(all-*E*)-β-carotene]	(430), 454, 478	536
30	(9*Z*)-β,β-carotene [(9*Z*)-β-carotene]	340, (426), 450, 474	536
31	(13*Z*)-β,β-carotene [(13*Z*)-β-carotene]	340, (424), 448, 472	536
32	(all-*E*)- or (*Z*)-7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene [(all-*E*)- or (*Z*)-phytofluene]	334, 350, 368	[e]
33	[(*Z*)- or (all-*E*)-phytofluene]	334, 350, 368	[e]
34	7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene (phytoene)	(276), 286, (295)	[e]

From Their Wavelengths of Absorption Maxima and Mass Spectral Data Determined by LC Photodiode Array Detection and Mass Spectrometry in the Order of Elution with Eluents B and A234.

[a] Refers to carotenoid metabolites.
[b] Common names for certain carotenoids are shown in parentheses.
[c] Values in parentheses represent points of inflection.
[d] The molecular ions appeared as the base peak (100% intensity). In some cases, the ion due to the loss of H_2O from the molecular parent ion (M) could also be observed.
[e] Owing to the coelution of cholestryl esters with this compound, its molecular parent ion was not observed by HPLC-MS.

Source: Reproduced with permission from, Khachik, F., Spangler, C. J., and Smith, J. C., Jr., *Anal. Chem.*, 69, 1873, 1997.

such as powders, beadlets or emulsions and tablets and capsules. The collaborative study[130] showed that variability within beadlet material introduced significant variation in subsampling. Also, weights of the outer shells of gel caps vary, which can introduce subsampling error. For extraction, water dispersible products, including beadlets, are digested with alkaline protease, and extracted with dichloromethane and alcohol. Oily suspensions are directly dissolved in dichloromethane and alcohol. Chromatography is isocratic reversed-phase (C_{18}) or, for products high in α-carotene, on C_{30} with gradient elution. The conclusion from the collaborative study[130] was that performance on beadlet raw material was unsatisfactory. Further, the Canadian Food Inspection Agency reported that the method underestimates β-carotene in microencapsulated materials.[285]

The European Committee for Standardization provides methodology for analysis of β-carotene in food in EN 12823-2 "Foodstuffs—Determination of Vitamin A by High Performance Liquid Chromatography—Part 2: Measurement of β-Carotene."[274] Samples are saponified, extracts are evaporated under vacuum, and the residue redissolved in mobile phase or other LC-compatible solvent. Reversed-phase chromatography, capable of resolving all-*trans*-β-carotene and *cis*-isomers, is used with detection at 450 nm. Method validation and precision are given in References 266 and 275, respectively.

Accurate quantitation and identification of carotenoids continues to be problematic. While HPLC, particularly with the C_{30} support, coupled with PDA detection and, often, interfaced with MS is a powerful analytical research tool, such instrument sophistication is not available in the majority of analytical laboratories worldwide. In addition, advantages of LC resolution are often negated by stability problems with the analytes that can cause the inexperienced analyst a great deal of difficulty. Scott[286] discussed specific problems associated with carotenoid analysis that all analysts should be aware of before initiating LC analysis. These include

1. Variation in results owing to inconsistencies in retention times, peak identification, and inconsistencies in peak homogeneity because of variations in mobile-phase composition and stationary supports.

2. On-column loss owing to degradation by interaction with stainless steel frits: Frits made of Peek Alloyed with Teflon (PAT) eliminate the problem.
3. Reaction of carotenoids with injection solvents and mobile phase: Failure to include an antioxidant such as BHT in the mobile phase and extraction solvent will intensify this problem. Also, low acid levels in solvents used for reversed-phase chromatography (e.g., methylene chloride) can be easily neutralized by addition of 0.001% TEA.[287]
4. Potential loss of labile carotenoids during solvent evaporation: inclusion of antioxidant in the extraction solvent tends to decrease degradation. Also, some methods call for the inclusion of a saturated hydrocarbon like decanoate in the solvent to protect labile analytes from oxidation during solvent evaporation. This can be particularly helpful if rotary evaporation is used. The hydrocarbon can form a thin film oxygen barrier on the rotary flask, protecting the carotenoids from low levels of oxygen that are potentially present in the evaporation system.
5. Potential for degradation during saponification (see Section 1.3.2.1.1).

Carotenoid identification from complex mixtures is still hampered by lack of reliable standards. Commercial, pure standards are limited. Craft and colleagues[288] evaluated all-*trans*-β-carotene sources by reversed-phase chromatography and found that impurities and *cis*-isomers accounted for 16%–75% of the absorbance at 450 nm. Analytical purity ranged from 7.1% to 82.9%. Probably, use of impure standards, failure to document purity of commercially available standards, and use of degraded standards will continue to be an impediment to reliable carotenoid data collection for the foreseeable future. We often learn the 'hard' way about stability of the carotenoids and associated assay problems.

Improved sensitivity of PDA detectors and much better software, use of multiple detection modes, and improved resolution systems have led to the use of methods for the simultaneous determination of multiple fat-soluble vitamins and their metabolites. These methods are discussed in Chapter 14.

1.4 Method protocols

Dried Skimmed Milk—Determination of Vitamin A—Colorimetric and Liquid Chromatographic Methods
Method B—High Performance Liquid Chromatographic Method
Bulletin of the IDF, 285, Chapter 8, 1993.[152]

Liquid Chromatographic Method

Principle

- Dried skimmed milk is saponified and extracted with light petroleum.
- Retinol is quantitated by reversed-phase LC with detection at 325 nm.

Chemicals

- Ethanol, 95%
- Sodium ascorbate solution, 200 g L^{-1}
- Potassium hydroxide, 50% in water wt/wt
- Potassium hydroxide, ethanolic; dissolve 3 g in water, add 10 mL ethanol, and dilute to 100 mL with water
- Light petroleum, boiling range 40°C–60°C or 60°C–80°C
- Methanol
- USP vitamin A—all-*trans*-retinyl acetate
- BHT

Apparatus

- Liquid chromatograph
- UV detector
- Saponification vessel fitted with reflux condenser
- Steam bath, boiling water bath, or electric heating mantle
- Water bath operating at a temperature up to 40°C

Procedure

Saponification

- Weigh 20 g sample (nearest 0.001 g) into beaker or conical flask; dissolve in 50 mL hot water (60–80°C). Cool to ambient temperature. Transfer to 100 mL volumetric and dilute with water.
- Transfer 25 mL of the solution to a saponification flask.
- Add 20 mL potassium hydroxide aqueous solution, 10 mL sodium ascorbate solution, and 50 mL ethanol, mix.
- Reflux 30 min on steam bath, water bath, or heating mantle.
- Swirl occasionally and cool immediately.

Extraction

- Transfer to separatory funnel with two 30-mL portions of water, two 10-mL portions of ethanol, and two 40-mL portions of light petroleum.
- Shake vigorously for 30 s; allow phase separation.
- Transfer aqueous phase to second separatory funnel, shake with a mixture of 10 mL ethanol and 40 mL light petroleum.
- Allow phase to separate, repeat extraction for a third time, add washings to the first separatory funnel.
- Shake the aqueous phase with 40 mL light petroleum and 10 mL ethanol; add the petroleum phase to the first separatory funnel.
- Wash the combined light petroleum extracts three times with 40-mL portions of ethanolic potassium hydroxide.
- Wash with 40-mL portions of water until washing is neutral.
- Dry extract by adding two sheets of 9-cm filter paper cut into strips.
- Transfer extract to 200 mL volumetric, add 10–20 mg BHT, dilute to volume with light petroleum.

Chromatography

Column	25 cm 34.6 mm
Stationary phase	C_8 or C_{18}, 10 mm
Mobile phase	Methanol:water (90:10 or ratio to accomplish resolution)
Flow rate	2 mL min^{-1}
Column temperature	Ambient
Injection	20 mL
Detection	325 nm
Calculation	Peak area, linear regression

Note: Even though the extraction procedure is based on multiple solvent extractions, the method has unacceptable repeatability and reproducibility characteristics. Stated limits were 14% for one analyst on the basis of two observations (repeatability), and 42% for two analysts working in different laboratories (reproducibility).

Evaluation of an LC Method for the Analysis of Carotenoids in Vegetables and Fruit
Food Chem., 59, 599, 1997.[201]

Principle

Carotenoids were extracted with methanol/THF (1:1) by homogenization. Following centrifugation, supernatants were, in some cases, saponified. After centrifugation or saponification, NaCl was added and carotenoids partitioned with petroleum ether. Organic layers were evaporated and the residue dissolved in methanol/THF (75:25). Carotenoids were resolved by reversed-phase chromatography with PDA detection.

Chemicals

- α-carotene, β-carotene
- β-cryptoxanthin, lutein, lycopene, zeaxanthin (standards)
- Ethyl-β-apo-8′-carotenate (IS)
- Potassium hydroxide
- Ethanol, absolute
- Methanol
- THF
- Hexane
- Acetone
- Petroleum ether
- BHT
- Sodium chloride
- Magnesium carbonate

Apparatus

- Liquid chromatograph fitted with PAT column frits
- UV detector
- Homogenizer
- Centrifuge
- Rotary evaporator
- Sonicator

Procedure

Extraction

- Add 0.2 g $MgCO_3$ to 0.5–1.0 g freeze-dried sample.
- Add ethyl-β-apo-8′-carotenoate (IS), amount approximately equal to carotenoids in sample.
- Homogenize with methanol/THF (1:1) until extract is colorless. Use 100 mL of extracting solution followed by 50 mL for subsequent extractions.
- Centrifuge to collect supernatants.
- If β-cryptoxanthin is present, extract was saponified at ambient temperature for 2 h with equal volume of 10% KOH.
- Add 50 mL 10% NaCl to extract (saponified or nonsaponified).
- Extract with 50 mL portions of petroleum ether until the petroleum ether is colorless.
- For saponified extract, wash petroleum ether with 100-mL portions of water until washes are neutral.
- Evaporate petroleum ether to dryness.
- Dissolve residue in methanol:THF (75:25) with sonication.

Chromatography

Column	25 cm 34.6 mm
Stationary phase	Vydac 201 TP, 5 mm
Mobile phase	Methanol:THF (95:5)
Flow rate	1 mL min^{-1}
Column temperature	Ambient
Detection	PDA, 450 nm
Calculation	Peak area, linear regression, internal standard

Notes

- $\%RSD_r$ ranged from 1.9% to 4.9%; recovery ranged from 93 to 107.
- Lycopene recovery is adversely affected by stainless steel frits in the pump. Replace with Peek Alloyed with Teflon (PAT).
- All solvents contain 0.1% BHT (w/v).

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chapter two

Vitamin D

2.1 Review

Rickets was first described in 1645. It was not until 1919 that rickets was experimentally induced in dogs by feeding fat-free diets and, then, cured by feeding cod liver oil.[1] Thus, rickets was recognized as a dietary deficiency of a fat-soluble factor. The antirachitic factor was named vitamin D by McCollum's research group in 1925 and, later that same year, the vitamin was proven to be produced in the skin by ultraviolet (UV) irradiation.[2] Vitamin D was isolated as Vitamin D_2 (ergocalciferol) from irradiated ergosterol from yeast in 1931, and its structure was identified in 1932. Vitamin D_3 (cholecalciferol) was structurally characterized in 1936 and was shown to be the antirachitic factor in cod liver oil.[1] The intermediary metabolism of the vitamin was more fully understood in the 1970s with the identification of the hydroxylated metabolites, 25-hydroxyvitamin D_3 (25(OH)D_3) and 1α,25-dihydroxyvitamin D_3 (1α,25(OH)$_2$$D_3$). Biologically, 1$\alpha$,25(OH)$_2$$D_3$ is the primary metabolically active form known as calcitrol. 1α,25(OH)$_2$$D_3$ and other hydroxylated, metabolically active forms such as 24R,25(OH)$_2$$D_3$ are produced in the kidney by the action of 25(OH)D_3-1-hydroxylase and 25(OH)D_3-24-hydroxylase. 25(OH)D_3 is primarily produced in the liver by D_3-25-hydroxylase.[3] Hepatic cytochrome CP-450s, mostly CYP2R1, are known to carry out hydroxylation of 25(OH)D_3.[1] Conversion of 25(OH)D_3 to 1α,25(OH)$_2$$D_3$ primarily takes place in the kidney.

The metabolic functions of vitamin D are accepted as that of a steroid hormone since vitamin D_3 must be converted to the biologically active hydroxylated forms. 1α,25(OH)$_2$$D_3$ is transported to target organs where receptor binding occurs. The cellular-receptor of 1α,25(OH)$_2$$D_3$ is referred to as the vitamin D receptor (VDR). After activation, VDR alters transcription rates of target genes responsible for the biological responses of 1α,25(OH)$_2$$D_3$.[4] The overall biological system is described as the vitamin D endocrine system.[3] Biological responses include mobilization and accretion of calcium and phosphorous (bone), calcium and phosphorous absorption (intestine), and reabsorption of calcium and phosphorous (kidney). Other accepted roles for vitamin D related to the vitamin D endocrine system include osteoblast formation, fetus development, pancreatic function, neural function, immunity, and 1α,25(OH)$_2$$D_3$ mediated cellular growth and differentiation effects. The vitamin D endocrine system is described by Norman,[3] and the reader is encouraged to refer to this review.

Vitamin D deficiency is rickets in infants and children and osteomalacia in adults. Deficiency due to inadequate intake or lack of exposure to sunlight responds to vitamin D therapy; however, vitamin D-resistant rickets do not. Vitamin D-resistant rickets results through genetic disorders and includes loss of the renal resorption system for phosphate, absence of 12-hydroxylase in the kidney, and through mutations in the VDR gene.[5]

Some types of vitamin D-resistant rickets can be treated with high doses of calcitrol. Documented cases of rickets are increasing to the extent that many health professionals are using the term 'epidemic.' Holick[6,7] attributes the increasing deficiency rate to an unappreciation of the beneficial effect of sunlight for health and to the lack of knowledge that human milk contains so little vitamin D that it does not satisfy the infant's requirement.

Vitamin D deficiency is prevalent in the elderly with inadequacy symptoms of muscle pain, fatigue, muscular weakness, and gait disturbances.[8,9] Severe vitamin D deficiency produces osteomalacea. Vitamin D inadequacy was found in 36% of young adults and in 57% of medical inpatients in the United States, indicating its widespread occurrence.[7] Holick attributes the high incidence of vitamin D inadequacy to low sunlight exposure, age-related decreases in synthesis, and diets low in vitamin D.[7] Almost all cases of inadequacy could be prevented through use of supplements and adequate sun exposure. Holick emphasizes that chronic vitamin D deficiency might lead to increased risk of hypertension, multiple sclerosis, colon, prostate, breast, and ovary cancers, and type 1 diabetes.[7]

Levels of vitamin D metabolites in serum and tissue are accepted status indicators for the human. Older indices include indirect measures of calcium and phosphorous levels in the serum and alkaline phosphatase activity. These measurements are considered nonspecific.[10] 25-Hydroxyvitamin D_3 in the serum is an accepted index of vitamin D status in the human owing to its long half-life.[10] It provides an estimate of vitamin D stores obtained from both synthesis and dietary intake over time.[10,11,12] Usual levels in the healthy adult range from 20 to 130 nmol L^{-1}.[10] Concentrations below 27.5 nmol L^{-1} (11 ng mL^{-1}) is accepted as an indicator of deficiency in infants and young children.[13] However, inadequate data exists to define 25(OH)D_3 levels necessary for maintenance of normal calcium metabolism and peak bone mass.[13]

Food sources of vitamin D are limited. Provitamin D compounds, however, are abundant in plant and animal tissue that can be converted to previtamin D compounds by UV irradiation. 7-Dehydrocholesterol (provitamin D_3) in animals is converted to previtamin D_3 by exposure of the skin to sunlight. Previtamin D_3 then undergoes isomerization to cholecalciferol (vitamin D_3). Vitamin D_3 concentration in animal tissue is dependent on dietary intake and exposure of the animal to sunlight. Formation through sunlight exposure provides humans with most of their vitamin D requirement.[12] Ergosterol (provitamin D_2) in plant tissue is converted to previtamin D_2 by irradiation. Previtamin D_2 isomerizes to ergocalciferol (vitamin D_2). The vitamin is limited in nature but is easily and cheaply synthesized. It is the primary synthetic form of vitamin D used for pharmaceuticals. The biological activities of vitamins D_2 and D_3 are approximately equal for humans. An International Unit (IU) of vitamin D is defined as the activity of 0.025 µg of cholecalciferol (vitamin D_3) measured by the rat or chick bioassay. The United States Pharmacopeial Convention (USP) reference standard for vitamin D is ergocalciferol (D_2) and/or cholecalciferol (D_3).

Sources of vitamin D include the vitamin D_3 synthesized in the body and food sources. Fortified fluid milk containing 10 µg (400 IU) per quart is a concentrated source of vitamin D_3. Although permitted in the United States, margarine fortification is not the rule as most margarines are not fortified. If added to margarine, the product must contain at least 1500 IU per pound (21 CFR 166).[14] Fortified breakfast cereals, fatty fish, eggs, butter, and some fish oils (cod liver oil) are sources of vitamin D. The Institute of Medicine considered data insufficient to accurately set an Estimated Average Requirement (EAR) necessary to establish a recommended dietary allowance (RDA). Therefore, adequate intake (AI) values were provided in the Dietary Reference Intakes (DRI) for vitamin D.[13] The AI levels range from 5 µg d^{-1} for infants through adults up to 50 years of age, 10 µg d^{-1} for 51–70 years, and 15 µg d^{-1} for individuals older than 70 years old (Table 2.1). The Reference Daily Intake (RDI) used for nutritional labeling is 10 µg or 400 IU.[15] The 2005 Dietary Guidelines for Americans[16] recommended that the elderly, people with dark skin, and those exposed to

Table 2.1 Dietary Reference Intakes and Tolerable Upper Intake Levels for Vitamin D

Life stage	DRI (μg d^{-1})	UL (μg d^{-1})
Infants (months)		
0–6	5	25
7–12	5	25
Children (years)		
1–3	5	50
4–8	5	50
Males (years)		
9–13	5	50
14–18	5	50
19–30	5	50
31–50	5	50
51–70	10	50
>70	15	50
Females (years)		
9–13	5	50
14–18	5	50
19–30	5	50
31–50	5	50
51–70	10	50
>70	15	50
Pregnancy (years)		
≤18	5	50
19–30	5	50
31–50	5	50
Lactation (years)		
≤18	5	50
19–30	5	50
31–50	5	50

Source: Food and Nutrition Board, Institute of Medicine, *Dietary Reference Intakes for Calcium, Phosphorous, Magnesium, Vitamin D and Fluoride*, National Academy of Sciences Press, Washington, DC, 1997, Chap. 7.

insufficient sunlight consume 25 μg d^{-1}. Johnson and Kimlin[9] gave the following guidelines for older adults to meet this recommendation:

1. Consume vitamin D-fortified foods, such as milk, daily.
2. Most older people will need dietary supplements that provide 20–25 μg (800–1000 IU) of vitamin D daily. Vitamin D_3 is the preferred source because of its superior bioavailability.
3. Assure older adults that they can take more than 100% of the daily value (DV) of 10 μg (400 IU) that is listed on the Supplement Facts panel.
4. Do not exceed the upper level (UL) for vitamin D of 50 μg (2000 IU) daily, unless under a physician's care.
5. Most older people do not consume enough calcium, so calcium supplements may also be needed.
6. Consult a registered dietitian and your physician for assistance with choosing a dietary supplement.

7. Do not recommend or self-prescribe additional sun exposure or artificial UV radiation to meet the vitamin D recommendation for older people. Instead, to reduce the risk of skin cancer, increase sun-protective behaviors such as avoiding the sun between 10 a.m. and 4 p.m., wearing sun-protective clothing when exposed to sunlight, using sunscreen with a sun protection factor (SPF) of 15 or higher, and avoiding artificial sources of UV radiation.

Tolerable upper intake levels (ULs) are set at 25 μg d^{-1} for infants up to 1 year of age and at 50 μg d^{-1} for the remainder of the population (Table 2.1).[13] Hypervitaminosis D leads to increases in serum 25(OH)D_3, which provides an indicator for toxicity diagnosis. Effects of hypervitaminosis D are characterized by hypercalcemia due to increased calcium absorption and resorption from bone.[13] In addition to hypercalcemia, symptoms include hypercalciuria, irreversible renal and cardiovascular damage, anorexia, vomiting, thirst, muscle weakness, joint pains, and disorentation.[3] It is difficult to reach intake levels necessary to cause hypervitaminosis D without misuse of supplements or processing errors that lead to overfortification.[17] Incidents of overfortification of fluid milk and infant formula have occurred and problems of variability in addition of vitamin D to the fluid milk supply have occurred.[18,19]

2.2 Properties

2.2.1 Chemistry

2.2.1.1 General properties

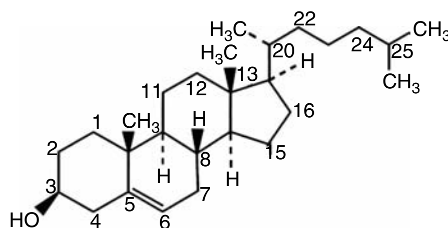
Vitamin D is the inclusive term for steroids that are antirachitic. Structures of cholecalciferol (vitamin D_3) and the steroid nucleus are given in Figure 2.1. International Union of Pure and Applied Chemistry–International Union of Biochemistry (IUPAC–IUB) nomenclature rules for steroid structure are used to characterize the ring system. The A, B, C, and D rings are derived from the cyclopentanoperhydrophenanthrene steroid structure with cholesterol serving as the parent compound.[21,22] Formation of previtamin D forms from the provitamins (7-dehydrocholesterol and ergosterol) requires opening of the B ring at the 9,10 bond. The vitamin D structures with the open ring are secosteroids. Accepted IUPAC–IUB systematic names are 9,10-seco(5Z,7E)-5,7,10(19) cholestatriene-3β-ol for vitamin D_3 and 9,10-seco(5Z,7E)-5,7,10(19),22 ergostate-traene-3β-ol for vitamin D_2. Conversions of the provitamins by irradiation to vitamin D_2 and vitamin D_3 along with the structures of the hydroxylated metabolites, 25(OH)D_3 and 1α,25$(OH)_2D_3$, are shown in Figure 2.2. Vitamin D_2 and vitamin D_3 differ structurally by only a double bond and an additional methyl group in the side chain at C-24 of vitamin D_2. Owing to the close structural similarity, their chemical and physical properties are similar (Table 2.2).[1,23,24]

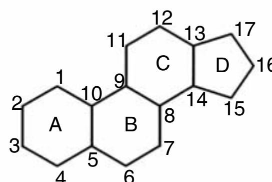
2.2.1.2 Spectral properties

All vitamin D forms show a characteristic broad UV spectrum with maximum absorption near 264 nm and a minimum near 228 nm.[1,25] Vitamin D does not fluoresce.

2.2.2 Stability

Vitamin D is stable in the absence of water, light, acidity, and at low temperatures. Isomerization to the 5,6-*trans*-isomer and isotachysterol[25] results under acid conditions or light exposure. The vitamin withstands alkalinity, and saponification is often used as the initial sample treatment for extraction of complex matrices. Oxidation can be a predominant route for decomposition at the conjugated double bonds at the 5,6 and 7,8 positions of the secosteroid structure; however, vitamin D is less susceptible to oxidative losses than vitamin E, β-carotene, and retinol.





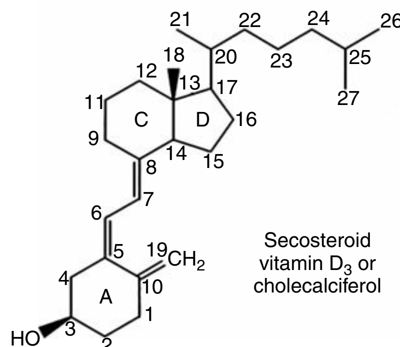


Figure 2.1 Structures of the steroid nucleus and vitamin D_3.

Various environmental conditions can cause isomerization of vitamins D_2 and D_3 to the previtamin D forms. Thermal interconversion is difficult to avoid completely during sample handling, and commercial concentrates and even standards can contain appreciable amounts of previtamin D. This fact requires that analytical methods account for all biologically active forms, including the previtamins, to accurately assess vitamin D activity. Tian and Holick[26] showed that isomerization of vitamin D_3 is shifted toward previtamin D_3 when complexed with β-cyclodextrin.

From a food-processing standpoint, vitamin D is quite stable. Because of the significance of fortified fluid milk and nonfat dry milk as vehicles for the delivery of vitamin D, its stability in these products has been documented. Renken and Warthesen[27] showed that light exposure caused only slight loss of vitamin D_3 in fortified milk. Air exposure did not affect the added vitamin. Indyk and colleagues[28] followed the processing steps for production of spray-dried, fortified whole milk. Thermal processing included preheating and direct steam injection to 95°C, five-stage evaporation, and spray drying at 149°C. The process did not produce statistically significant losses of vitamin D_3. Min and colleagues have

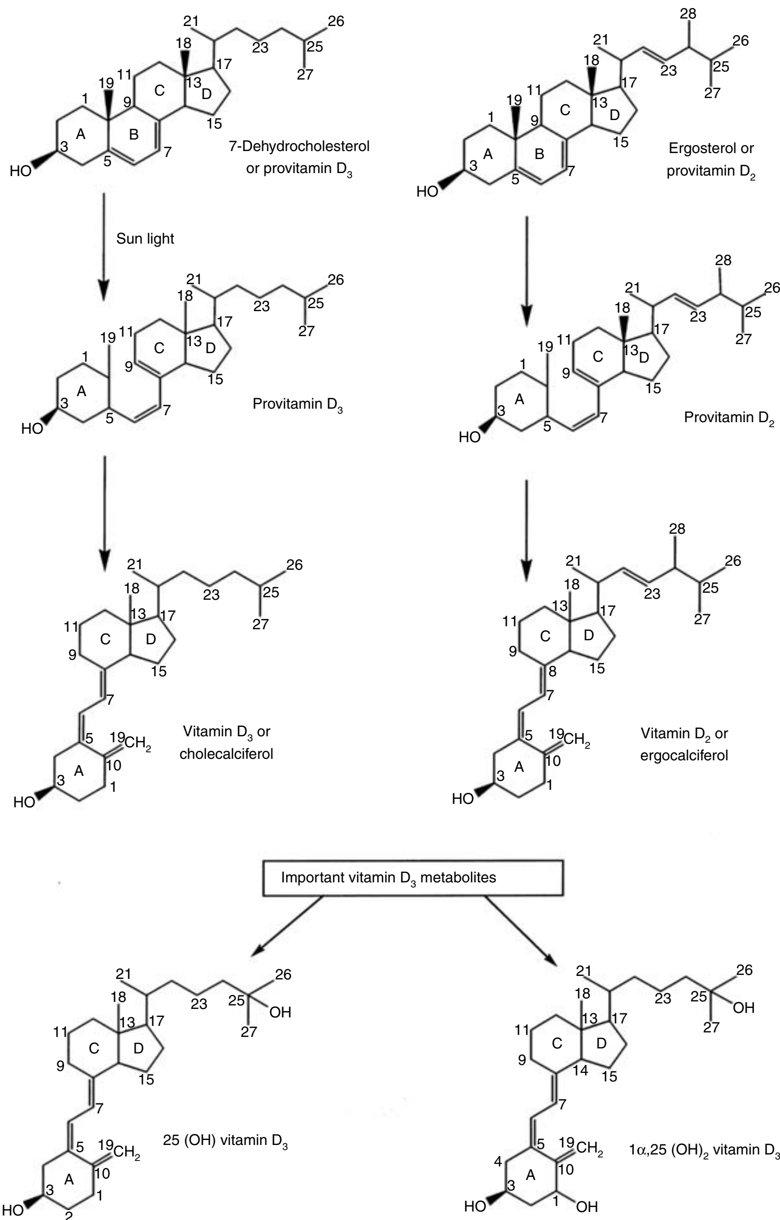


Figure 2.2 Structures of the vitamin D_3 and D_2 precursors and metabolites.

studied in-depth the riboflavin-photosensitized singlet oxygen oxidation of vitamin D_2. Their studies showed that a 5,6-epoxide of vitamin D_2 was formed in the presence of riboflavin under illumination at 4000 lux. They concluded that milk should not be exposed to light during processing and storage. Riboflavin increased oxidation of vitamin D_2 by singlet oxygen under light but not under dark.[29–31]

Stability of vitamin D_3 in muscle foods is quite variable depending on the processing method. Clausen and colleagues[32] reported no loss in vitamin D_3 in over-roasted pork cuts. Cooked beef retained 58–65% of vitamin D_3 depending on the beef cut.[33] Solar drying completely destroyed vitamin D_3 in mackerel and saury.[34] Production of refined, bleached, and deodorized (RBD) menhaden oil decreased vitamin D_3 levels by 80%.[35]

Table 2.2 Physical Properties of Vitamin D and Hydroxylated Forms

Substance[a]	Molar mass	Formula	Solubility	Melting point (°C)	Crystal form	Spectral properties		
						λ_{max} (nm)	$E^{1\%}_{1\,cm}$	$\varepsilon \times 10^{-3}$
D_3 CAS No. 67-97-0 **10157**	384.65	$C_{27}H_{44}O$	Soluble in most organic solvents Insoluble in water	84–85	Fine needles Yellow to white	264	485	18.3
D_2 CAS No. 50-14-6 **10156**	396.66	$C_{28}H_{44}O$	Soluble in most organic solvents Insoluble in water	115–118	Prisms Yellow to white	264	462	19.4
$25(OH)D_3$ **1677**	400.65	$C_{27}H_{44}O_2$	Soluble in most organic solvents Insoluble in water	82–83		265	[449][b]	18.0
$1\alpha,25(OH)_2D_3$ **1681**	416.64	$C_{27}H_{44}O_3$	Slightly soluble in methanol, ethanol and ethylacetate Insoluble in water	111–115	White crystalline powder	264	[418][b]	19.0

[a] Common or generic name; CAS No.: Chemical Abstract Service number; bold print designates the Merck Index Monograph number.

[b] Values in brackets are calculated from corresponding ε value, in ethanol.

Sources: Friedrich, W., In *Vitamins*, Walter de Gruyter, Berlin, 1998, Chap. 3; Budavari, S., *The Merck Index*, 13th ed., Merck and Company, Whitehouse Station, New Jersey, 2001; DeLuca, H. F., *Vitamin D*, *The Fat-Soluble Vitamins*, Plenum Press, New York, 1978, Chap. 2.

2.2.3 Bioavailability

Ingested vitamin D is incorporated into the chylomicrons following enterocyte uptake and transported by the lymphatic system.[36] However, relatively little is known about the bioavailability from foods or supplements. van den Berg[36] in an excellent review of the bioavailability of vitamin D gives the following information:

1. 25(OH)D absorption is faster and more efficient than absorption of vitamin D.
2. Oral intake of 25(OH)D is approximately ten times as effective in increasing serum 25(OH)D levels compared to vitamin D.
3. A high fiber diet may decrease bioavailability.
4. Absorption from supplements varies between 55% and 99%, and absorption from dietary sources is probably lower. Bioavailability from beef was 60% compared to a supplemental source.[37]
5. Bioavailability studies are complicated by the contribution of vitamin D produced by sunlight exposure. Validated procedures to measure bioavailability of vitamin D are unavailable.

Since most foods that contain vitamin D also contain 25(OH)D_3, measurement of 25(OH)D_3 levels together with use of a proper conversion factor to account for the more efficient absorption of 25(OH)D_3 would provide a better estimate of vitamin D activity.[38] The proper conversion factor is not known but has been estimated to be 1.5–5.[38]

In a recent study by Natri and colleagues,[39] addition of vitamin D_3 to bread proved to be a feasible fortification approach. The vitamin was stable and fortified wheat and rye breads increased serum 25(OH)D_3 concentration to levels comparable to use of a vitamin D_3 supplement.

2.3 Methods

2.3.1 General approach

Methods for assay of vitamin D in foods, clinical samples, vitamin premixes, concentrates, and pharmaceuticals are varied in the chemical or biological approach depending on the matrix and concentration level. Available procedures are colorimetric, spectrophotometric, chromatographic, immunological, ligand binding, or biological in approach. Excellent methods based on competitive protein binding assays (CPBAs) and radioreceptor assay (RRA) using [^{3}H]$^-$ metabolites have been available for the past several decades for the analysis of serum.[40] These methods rely upon extensive prepurification of extracts by liquid chromatography (LC) or solid-phase chromatography. Methodology shown in Figure 2.3 that incorporates LC for vitamin D_2, vitamin D_3, 25(OH)D_2 and -D_3, CPBA for 24,25$(OH)_2D_2$ and -D_3, and RRA for 1α,25$(OH)_2D_2$ and -D_3.[41] This approach combines currently accepted assay methods for serum analysis.

CPBA and RRA are specific and sensitive enough to measure 1α,25$(OH)_2$D at circulating levels less than 100 pg mL^{-1}. However, the extensive extract purification and use of [^{3}H]-labeled compounds has been an impediment to general clinical use.[42] This situation led to the development of radioimmunoassays (RIAs) using ^{125}I-labeled metabolites and the elimination of the extensive cleanup and concentration steps. Methods for 25(OH)D and 1α,25$(OH)_2$D require little sample preparation and use serum calibrators that allow direct quantitation without the need for recovery determinations for each sample.[42,43] RIA analysis of serum includes the following steps:

1. Preparation of assay calibrators by stripping 1α,25$(OH)_2$D with activated charcoal and addition of crystalline 1α,25$(OH)_2D_3$ at concentrations of 0, 5, 10, 20, 50, 100, and 200 ng L^{-1}

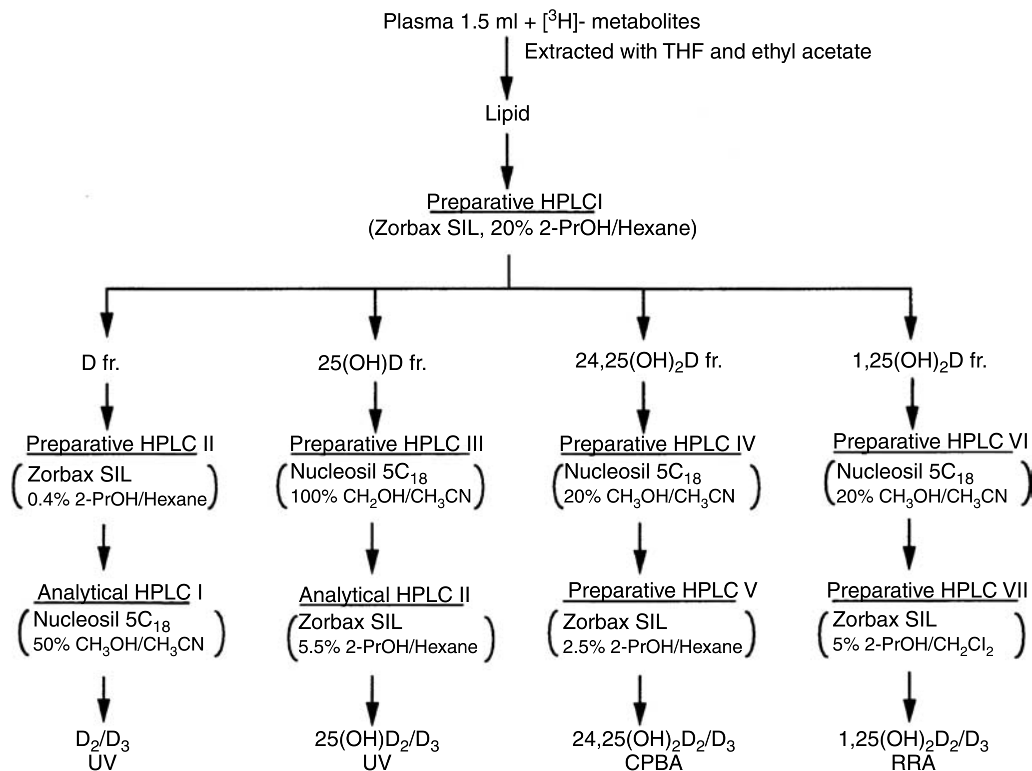


Figure 2.3 Procedure for determination of vitamin D_2, vitamin D_3, and hydroxylated metabolites in serum. (Reproduced with permission from Masuda, S., Okano, T., and Kobayashi, T., *Food Chem.*, 45, 215, 1992.)

2. Extraction of serum with acetonitrile
3. Pretreatment of the extract with sodium periodate to convert 24,25$(OH)_2D_3$ and 25,26$(OH)_2D_3$ into aldehydes and ketones to facilitate their removal by subsequent solid phase chromatography
4. Isolation of 1α,25$(OH)_2$D by two successive C_{18}–OH silica cartridge chromatography passes
5. RIA

The assay can be completed within 5 h with a detection limit of 2.4 ng L^{-1}. Results compare with accepted RRA techniques.[43] Incorporation of the RIA procedures will dramatically change the operation of clinical laboratories for assay of 25(OH)D and 1α,25$(OH)_2$D.

Continued improvement of LC instrumentation and supports and recently improved LC/MS technology has greatly improved both resolution and sensitivity of available assays. LC coupled to mass spectrometry (MS) has been advantageously used over the past decade for both food and clinical sample analysis. New LC procedures are discussed in Section 2.

Older methods not based on LC, CPBA, RRA, or RIA were reviewed by Singh.[44] Reviews on the use of LC for food and clinical sample analysis include Porteous et al.[45] Norman et al.,[46] Eitenmiller and Landen,[47] Jones and Makin,[48] Collins and Norman,[49] Luque de Castro et al.,[50] Zerwakh,[11] Ye and Eitenmiller,[51] and Perales et al.[52]

2.3.2 Regulatory and handbook methods

Various regulatory and handbook methods for the analysis of vitamin D are summarized in Table 2.3.[53–72] However, as is the case for many areas of vitamin assay, these methods have

Table 2.3 Regulatory and Handbook Methods for the Analysis of Vitamin D

Source	From	Methods and application	Approach	Most current crossreference
***U.S. Pharmacopeia, National Formulary*, 2006, USP 29/NF 24 Dietary Supplements Official Monographs**[53]				
1. Pages 2388, 2389	Ergocalciferol Cholecalciferol	Vitamin D in oil-soluble vitamin capsules/tablets	LC 265 nm	None
2. Pages 2391, 2394–2395, 2397, 2402, 2409–2410	Ergocalciferol Cholecalciferol	Vitamin A in oil– and water-soluble vitamin capsules/tablets, oral solution w/wo mineral	LC 265 nm	None
3. Pages 502	Cholecalciferol	Cholecalciferol (NLT 97.0%, NMT 103.0%)	LC 254 nm	None
4. Pages 812–813	Ergocalciferol	Ergocalciferol (NLT 97.0%, NMT 103.0%)	LC 254 nm	None
5. Pages 813–814	Ergocalciferol	Ergocalciferol capsules	LC 254 nm	None
6. Pages 814–815	Ergocalciferol	Ergocalciferol oral solution	LC 254 nm	None
7. Page 815	Ergocalciferol	Ergocalciferol tablets	Colorimetric 500 nm	None
***British Pharmacopoeia*, 2007**[54]				
1. Pages 577–579	Cholecalciferol	Vitamin D_3 (NLT 97.0%, NMT 103.0%)	LC 254 nm	None
2. Pages 579–581	Cholecalciferol	Vitamin D_3 concentrate (oily form)	LC 254 nm	None
3. Pages 581–583	Cholecalciferol	Vitamin D_3 concentrate (powder form)	LC 254 nm	None
4. Pages 583–585	Cholecalciferol	Vitamin D_3 concentrate (water-dispersible form)	LC 254 nm	None
5. Pages 2469–2470	Cholecalciferol	Vitamin D_3 injection	Spectro-photometric	None
6. Page 2470	Cholecalciferol	Vitamin D_3 tablets	Spectrophoto-metric or LC 254 nm	None
7. Pages 2987–2989	Cholecalciferol	Pediatric vitamins A, C, and D oral drops	LC 254 nm	None
8. Pages 773–774	Ergocalciferol	Vitamin D_2 (NLT 97.0%, NMT 103.0%)	LC 254 nm	None
9. Page 2546	Ergocalciferol	Vitamin D_2 injection	Spectrophoto-metric	None
10. Pages 2546–2547	Ergocalciferol	Vitamin D_2 tablets	Spectrophoto-metric	None
***AOAC Official Methods of Analysis*, 18th ed., 2005**[55]				
1. 45.1.17	Ergocalciferol Cholecalciferol	AOAC Official Method 975.42 Vitamin D in vitamin preparations (200–20,000,000 IU g^{-1})	Colorimetric 550 nm	*J. Assoc. Off. Anal. Chem.*, 60, 151, 1977[56]
2. 45.1.18	Ergocalciferol Cholecalciferol	AOAC Official Method 979.24 Vitamin D in vitamin preparations (25,000–20,000,000 IU g^{-1})	LC 254 nm	*J. Assoc. Off. Anal. Chem.*, 64, 58, 1981[57]

Table 2.3 (Continued)

Source	From	Methods and application	Approach	Most current crossreference
***AOAC Official Methods of Analysis*, 18th ed., 2005**[55](*Continued*)				
3. 45.1.19	Ergocalciferol Cholecalciferol	AOAC Official Method 980.26 Vitamin D in multivitamin preparations (≥ 200 IU g^{-1})	LC 254 nm	*J. Assoc. Off. Anal. Chem.*, 64, 61, 1981[58]
4. 45.1.20	Ergocalciferol Cholecalciferol	AOAC Official Method 985.27 Vitamin D in vitamin AD concentrates (≥5,000 IU g^{-1})	LC 254 nm	*J. Assoc. Off. Anal. Chem.*, 68, 822, 1985[59]
5. 45.1.21	Ergocalciferol Cholecalciferol	AOAC Official Method 981.17 Vitamin D in fortified milk and milk powder (≥1 IU g^{-1})	LC 254 nm	*J. Assoc. Off. Anal. Chem.*, 65, 1228, 1982[60]
6. 45.1.22	Ergocalciferol Cholecalciferol	AOAC Official Method 982.29 Vitamin D in mixed feeds, premixes and pet foods (>2 <200 IU g^{-1}) (<200 IU g^{-1})	LC 254 nm	*J. Assoc. Off. Anal. Chem.*, 66, 751, 1983[61]
7. 45.1.22A	Cholecalciferol	AOAC Official Method 2002.05 Vitamin D_3 in selected foods (not containing vitamin D_2)	LC 265 nm	*J. AOAC Int.*, 86, 400, 2003[62]
8. 45.3.02	Ergocalciferol Cholecalciferol	AOAC Official Method 936.14 Vitamin D in milk, vitamin preparations and feed concentrates	Rat bioassay	*Anal. Chem.*, 24, 1841, 1954;[63] *J. Assoc. Off. Anal. Chem.*, 46, 160, 1963[64]
9. 45.3.03	Cholecalciferol	AOAC Official Method 932.16 Vitamin D_3 in poultry feed supplements	Chick bioassay	*J. Assoc. Off. Anal. Chem.*, 51, 591, 1968[65]
10. 50.1.05	Cholecalciferol	AOAC Official Method 992.26 Vitamin D_3 (cholecalciferol) in Ready-to-Feed Milk-Based Infant Formula (488–533 IU L^{-1})	LC 254 nm	*J. AOAC Int.*, 76, 1042, 1993;[66] *J. Assoc. Off. Anal. Chem.*, 68, 177, 1985[67]
11. 50.1.23	Cholecalciferol Ergocalciferol	AOAC Official Method 995.05 Vitamin D in infant formula and enteral products	LC 265 nm	*J. AOAC Int.*, 75, 566, 1992;[68] 79, 73, 1996[69]
***Food Chemicals Codex*, 5th ed., 2004**[70]				
1. Pages 497–498	Ergocalciferol	Vitamin D_2 (NLT 97.0%, NMT 103.0%)	LC 254 nm	None
2. Pages 498–499	Cholecalciferol	Vitamin D_3 (NLT 97.0%, NMT 103.0%)	LC 254 nm	None
International Dairy Federation, IDF Standard 177, 1996[71]				
	Cholecalciferol Ergocalciferol	Determination of vitamin D_2 or D_3 in dried skimmed milk	LC 265 nm	None
European Committee for Standardization[72]				
1. EN 1282172	Cholecalciferol Ergocalciferol	Foods	LC 265 nm	*J. Food Compos. Anal.*, 5, 281, 1992;[73] *Int. J. Vit. Nutr. Res.*, 57, 357, 1987[74]

not kept abreast of methods developed for research either in the food or in the clinical fields.

2.3.2.1 AOAC International

Association of Official Analytical Chemists (AOAC) International Method 936.14 (45.3.02) "Vitamin D in Milk, Vitamin Preparations, and Feed Concentrates, Rat Bioassay" is the often quoted and still used line test.[55] Weanling rats are depleted of vitamin D over a 16-to 25-day depletion course and, after depletion, standard vitamin D doses and test samples are fed for 7–12 days. Recalcification of the tibia or distal end of a radius or ulna is determined by staining newly calcified areas with silver nitrate. Length and width of the calcification line determines potency of the sample. The assay is sensitive with detection limits as low as one IU of vitamin D. The assay is time-consuming and expensive, and few laboratories worldwide have maintained the capability to conduct the assay. In the United States, the Center for Food Safety and Nutrition (CFSAN), Food and Drug Administration, maintains the capability. AOAC International Method 932.16 (45.3.03) "Vitamin D_3 in Poultry Feed Supplements, Chick Bioassay" specifically is used for analysis of vitamin D_3 in supplements for poultry rations. Vitamin D_3 is ten times more potent than vitamin D_2 for bone development in the chick.[49] Vitamin D deficient diets are fed to newly hatched chicks with standard doses of vitamin D_3. Bone ash of the tibia is assayed after 3 weeks.

AOAC Method 975.42 "Vitamin D in Vitamin Preparations" is an older, colorimetric method for concentrates and multivitamins based on open-column chromatography. This method is not frequently used. The method has been replaced with AOAC Method 985.27 "Vitamin D in Vitamin AD Concentrates," which is LC-based and applicable to oil, powder, and aqueous dispersion AD concentrates containing ≥5000 IU vitamin D g^{-1}.

Older AOAC methods for vitamin D using LC include Method 979.24 "Vitamin D in Vitamin Preparations," Method 980.26 "Vitamin D in Multivitamin Preparations," Method 981.17 "Vitamin D in Fortified Milk and Milk Powder" and Method 982.29 "Vitamin D in Mixed Feeds, Premixes, and Pet Foods." These methods are suitable only for concentrated preparations or fortified foods.

AOAC Method 992.26 "Vitamin D_3 (Cholecalciferol) in Ready-To-Feed Milk-Based Infant Formula" was based on the work of Sertl and Molitor[67] published in 1985. The method was collaborated by AOAC International in 1993[66] declared First Action in 1992 and Received Final Action in 1995. Procedural steps include saponification, extraction with ethyl ether and petroleum ether (1:1), LC cleanup on a Partasil-5 PAC column, and determinative LC on 3-μm Apex silica. Mobile phases for the cleanup and analytical columns were 0.8% amyl alcohol in hexane and 0.15% amyl alcohol in hexane, respectively. Cleanup provided by the Partisil-5 PAC (amino-cyano) column was insufficient to provide clean chromatograms by analytical chromatography. Vitamin D was quantitated on the slope of a large interfering peak. Of note, most cleanup procedures utilize chromatography modes of different separation chemistries compared to the analytical system. In order to maximize cleanup, reversed-phase supports are used before determinative chromatography on normal-phase supports or vice versa. While the method was accepted by AOAC International as official, RSD_r and RSD_R values were greater than 20%, an unacceptably high degree of method performance variability.

Sliva and colleagues[68] presented a method for vitamin D in infant formula and enteral nutritionals that was recently collaborated by AOAC International[69] in 1996. AOAC Method 995.05 "Vitamin D in Infant Formulas and Enteral Products" received First Action in 1995 and incorporates saponification, hexane extraction, SPE on silica, and analytical chromatography on C_{18}. The procedure provides RSD_r values of 13.48 and 9.44, respectively, which is a significant improvement over Method 992.26.[68,69] Chromatograms showed excellent resolution of vitamin D_2 (internal standard), vitamin D_3, and previtamins D_2 and D_3. Levels of

previtamins were below detection limits following the saponification at 60°C for 30 min, indicating excellent control during the extraction.

AOAC Method 2002.05 "Cholecalciferol (Vitamin D_3) in Selected Foods" received First Action in 2002. Staffas and Nyman[62] of the Swedish National Food Administration presented the collaborative study, which was completed by the Nordic Committee on Food Analysis (NMLK). The procedures were based on the method developed by Johnson and Hessel[74] in 1987. The method uses vitamin D_2 as the internal standard and is applicable to foods containing only vitamin D_3. After addition of the internal standard, the samples are saponified and extracted with *n*-heptane. The vitamin D_2/D_3 fraction is isolated and concentrated by normal-phase liquid chromatography. Quantitation is on a reversed-phase system with UV detection at 265 nm. The method was tested with six food matrices by eight participating laboratories. Recoveries ranged from 93% to 102% and RSD_r values ranged from 2.2% to 7.4%. RSD_R values ranged from 6.8% to 24.0%.

2.3.2.2 European Committee for Standardization

AOAC Method 2002.05 was declared First Action after acceptance of the procedure by the European Committee for Standardization, which approved the procedure submitted by their Technical Committee (CEN/TC275) in 2000. Method EN12821[72] "Foodstuffs—Determination of vitamin D by high performance liquid chromatography—Measurement of cholecalciferol (D_3) and ergocalciferol (D_2)" allows use of vitamin D_2 as the internal standard for quantitation of vitamin D_3 and use of vitamin D_3 as the internal standard for quantitation of vitamin D_2.

2.3.2.3 International Dairy Federation

In 1996, the International Dairy Federation (IDF) published IDF Standard 177: 1996 "Dried Skimmed Milk, Determination of Vitamin D Content."[71] The method was chosen after results of an intercomparison study of methods by the Measurements and Testing Program of the Community Bureau of Reference (BCR) of the European Commission.[126] The procedure is based upon saponification, exhaustive partitioning of the digest with light petroleum, semipreparative LC on silia, and analytical LC on C_{18} or ODS. The limit of detection is 2.5 μg 100 g^{-1}. Vitamin D_2 is used as the internal standard for determination of vitamin D_3. However, the absence of vitamin D_2 at detectable levels must be verified by assaying the sample with and without addition of the internal standard.

2.3.3 High performance liquid chromatography

High performance liquid chromatography (HPLC) methods are available for the quantitation of vitamin D from most matrices. Further, it has been the primary tool for purification and concentration of hydroxylated metabolites before quantitation by more sensitive CPBA and RRA techniques (Section 2.3.1). Selected LC methodology papers are provided in Table 2.4.[62,66–68,72,73,75–125]

2.3.3.1 Extraction procedures for analysis of vitamin D by liquid chromatography

As shown in Table 2.4, saponification has been used almost exclusively as the initial extraction step for analysis of vitamin D in foods. In order to concentrate and clean the extract from the saponified digest, solid-phase extraction (SPE) gel permeation chromatography and semipreparative LC have been used with good success. Methods presented by Kurman and Indyk,[84] Konings[86] and Mattila and colleagues[72,88–94] applied semipreparative LC to a wide variety of fortified and nonfortified foods before determinative chromatography. Recent work by Ostermeyer and Schmidt[98] used normal phase semiprep LC on LiChrosorb Si60 following EN12821.[72] The procedure was used to assay vitamin D content of 38 fish

Table 2.4 LC and LC-MS Methods for the Analysis of Vitamin D and Its Metabolites in Foods, Pharmaceuticals, Feed, and Biologicals

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods					
Milk/D_2, D_3	Saponification, ambient, overnight. Extract w/Et_2O Clean-up: Hydroxyalkoxypropyl Sephadex	LiChrosorb Si 60 5 µm, 25 cm × 3.2 mm Mobile phase—isocratic 0.6% IPA in Hex (50% water saturated) Flow rate—1 mL min^{-1}	265 nm	QL—0.08 µg 100 mL^{-1} %Recovery >97	*J. Assoc. Off. Anal. Chem.*, 60, 998, 1977[75]
Fortified milk, margarine, infant formula/D_2, D_3	Saponification, ambient, overnight. Extract w/Hex Clean-up: Infant formula—Silica Margarine—Alumina	Spherisorb ODS, 10 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:MeCN (1:9) Flow rate—1 mL min^{-1}	265 nm	—	*J. Assoc. Off. Anal. Chem.*, 65, 624, 1982[76]
Whole milk powder/D_2, D_3	Saponification, ambient, overnight. Extract w/PE:Et_2O (90:10)	Two RCM100 units w/ Rad-PAK C_{18} 5 µm, 8 mm ID^{-1} Mobile phase—isocratic MeOH (100%) or MeOH:THF (99:1)	Dual wavelength 250 nm 280 nm	QL—30 IU $100g^{-1}$ %Recovery—95	*NZ J. Dairy Sci. Technol.*, 19, 19, 1984[77]
Fortified milk infant formula/D_2, D_3	Homogenize w/IPA-CH_2Cl_2 w/$MgSO_4$ to remove water. Evaporate, dissolve in CH_2Cl_2. Clean-up: a. Gel permeation chromatography 4 µ styragel (100 Å) in series b. µ Bondapak/NH_2	Zorbax ODS 6 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:CH_2Cl_2:MeOH (700:300:2) Flow rate—1 mL min^{-1}	Dual wavelength 280 nm 254 nm	%Recovery—89.6 %CV—7.5	*J. Assoc. Off. Anal. Chem.*, 68, 183, 1985[78]
Milk, infant formula/D_2, D_3	Saponification, 75°C, 30 min. Extract w/Et_2O. Evaporate, redissolve in Hex Clean-up: Pertisil—5 PAC, 25 cm × 4.6 mm	Apex silica 3 µm, 15 cm × 4.5 mm Mobile phase—isocratic Hex:amyl alcohol (100:0.15) Flow rate—3 mL min^{-1}	254 nm	QL—100 IU L^{-1} %RSD—7.7	*J. Assoc. Off. Anal. Chem.*, 68, 177, 1985;[67] *J. AOAC Int.*, 76, 1042, 1993[66]
Milk powder, infant formula/D_2, D_3	Saponification, add ^3H-D_3 and $^3[H]$-25 $(OH)D_3$ for recovery, steam bath, 45°C. Evaporate, dissolve in MeOH containing 1.5% digitonin. Extract w/Hex Clean-up: Aluminum oxide	Polygosil Si 60-5, 25 cm × 4.6 mm Mobile phase—isocratic 0.5% IPA in Hex	CPBA	QL—0.1 IU g^{-1} %Recovery—90–110	*J. Agric. Food Chem.*, 34, 264, 1986[79]

Fortified milk, infant formula, porridge, gruel/D_3	Saponification, boiling water bath, 60 min, extract w/B Clean-up: Polygosil Si 60-5, 30 cm × 8.0 mm, monitor at 254 nm	Two Shandon ODS columns in series, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH	265 nm	QL—5 µg L^{-1} %Recovery—95	*Int. J. Vit. Nutr. Res.*, 57, 357, 1987[74]
Human milk, infant formula/D_3	Add ^{3}[H]-D_3 for recovery. Deproteinize w/NH_4OH and ethanol. Extract w/EtOH and PE. Evaporate to dryness and saponify, 50°C, 15 min. Extract w/PE Clean-up: (a) Sep-Pak silica (b) LC cleanup µBondpak C_{18}, 10 µm, 36 cm × 3.9 mm	—	CPBA	QL—2 µg L^{-1} %Recovery—65–75	*Clin. Chem.*, 33, 796, 1987[80]
Fortified milk, milk powder, infant formula/D_2, D_3	Saponification, ambient, overnight. Extract w/I-chlorobutane, evaporate. Redissolve in 2,2,4-trimethylpentane Clean-up: Polygosil 60-5 CN 12.5 cm x 4 mm	Polygosil 60-5 NH_2CN 12.5 cm × 4 mm Mobile phase—isocratic 0.35% I-pentanol in Hex Flow rate—1 mL min^{-1}	254 nm 265 nm 280 nm	%Recovery—94.8	*Neth. Milk. Dairy J.*, 42, 423, 1988[81]
Infant formula, enteral nutritionals/ D_2, D_3	Add D_2 (IS), saponification, 60°C, 30 min. Extract w/Hex, evaporate Clean-up: SPE B & J Silica 9054 Evaporate, redissolve in MeCN	Vydac 201TP54 5 µm, 25 cm × 4.6 mm (not endcapped) Mobile phase—gradient MeCN–MeOH–EtOAC Flow rate—0.7–2.5 mL min^{-1}	265 nm	QL—8 IU $quart^{-1}$ %Recovery—99 %RSD_R—13.48	*J. AOAC Int.*, 75, 566, 1992;[68] 79, 73, 1993[69]
Meat, animal fat/ D_3, 25(OH)D_3	Saponification. Extract w/Hex: CH_2Dl_2 (85:15). Evaporate, redissolve in Hex. No IS Clean-up: (a) Alumina, open-column (b) HPLC (Apex Silica) for D_3	RCM module, resolve 8C18 5 µm Mobile phase—isocratic MeOH, 100% Flow rate—1.5 mL min^{-1}	254 nm	—	*Food Chem.*, 46, 313, 1993[82]
Milk/D_2, D_3	Add D_2 (IS), saponification, 60°C, 30 min. Extract w/Hex, evaporate, and redissolve in Hex Clean-up: SPE Florisil Sep-Pak, evaporate, redissolve in MeCN	Vydac C_{18} 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:MeOH (97:3) Flow rate—1 mL min^{-1}	265 nm	QL—1.6 µg L^{-1} %Recovery—81–96	*J. AOAC Int.*, 77, 1047, 1994[83]

Continued

Table 2.4 (Continued)

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods (*Continued*)					
Milk/D_2, D_3	Add D_2 (IS), saponification, ambient, overnight. Extract w/Hex:Et_2O (9:1). Evaporate, redissolve in Hex Clean-up: (a) SPE (b) Semiprep LC, resolve silica, 5 µm, 8 × 10 RCM	Rad-Pak, Resolve C_{18}, 5 µm 8 × 10 RCM, at 30°C Mobile phase—isocratic MeOH:THF:water (93:2:5) Flow rate—1 mL min^{-1}	280 nm 265 nm PDA	QL—0.06 IU fat g^{-1} %RSD_R—2.7 %Recovery >80	*Food Chem.*, 50, 75, 1994;[84] 57, 283, 1996[85]
Baby foods/D_2, D_3	Add D_2 or D_3 (IS). Saponification. Extract w/Et_2O:PE (1:9), evaporate and redissolve in iOct Clean-up: Semiprep LC, Polygosil-60—5 µm, 25 ×m × 8 nm	Two Shandon ODS-5 columns in series 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH (100%) Flow rate—1 mL min^{-1}	265 nm	QL—350–420 IU kg^{-1} %Recovery—84 DL (on-column)—2 ng.	*Neth. Milk Dairy J.*, 48, 31, 1994[86]
Fish/D_3, 7-dehydro-cholesterol (7-DHC), 25(OH)D_3	Add ^{3}[H]-D_3 and 25-OH^3[H]-D_3 for recovery determination. Saponification, extract w/B. Evaporate, redissolve in MeOH:Hex:$CHCl_3$ (2:23:75) Clean-up: Sephadex LH-20	(a) Zorbax ODS 5 µm, 15 cm × 4.6 mm Mobile phase—isocratic MeOH:MeCN (50:50) Flow rate—2 mL min^{-1} (b) Zorbax—SIL 5 µm, 15 cm × 4.6 mm Mobile phase—isocratic Hex:IPA (different ratio for different analytes.	265 nm	%Recovery—95–98	*Comp. Biochem. Physiol.*, 111A, 191, 1995[87]
Fish, fish products, egg yolk, mushroom, meat, liver, milk products, foods/D_2, D_3, 25(OH)D_3	Add D_2 (IS) and 25(OH)D_3 (IS). Saponification, extract w/Hex, evaporate and redissolve in Hex. Clean-up: (a) SPE Mega Bond Elut Silica (b) Semiprep LC µPorasil, 10 µm, 30 ×m × 3.9 mm	Vydac 201TP54 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:water (83:17) Flow rate—1 mL min^{-1}	264 nm	DL (on-column)—0.5–0.7 ng QL—0.4 µg $100g^{-1}$ %Recovery—94 %CV—3.6	*J. Food Compos. Anal.*,5, 281, 1992;[73] 6, 250, 1993[88] & 8, 232, 1995;[89] *J. Agric. Food Chem.*, 42, 2449, 1994;[90] 43, 2394, 1995[91] & 45, 3891, 1997;[92] *Food Chem.*, 57, 95, 1996;[93] *J. Sci Food Agric.*, 79, 195, 1999[94]

Animal products/ D_3, 25(OH)D_3	Saponification. Extract w/ether: PE (1:1). Clean-up: SPE, silica and semiprep LC	C_{18} (Vydac) for D_3 w/mobile phase—MeCN:MeOH (80:20) and C_{18} (Luna) for 25(OH)D_3 w/mobile phase—MeOH: water (90:10) Flow rate—1 mL min^{-1}	265 nm	%CV—7	*J. Food Compos. Anal.*, 16, 575, 2003[95] & 17, 777, 2004.[96]
Foods/D_2, D_3	Saponification, extract heptane. Clean-up: Prep NP LC	Vydac 201 TP54 C_{18} 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:MeCN (20:80)	265 nm	%Recovery—93–102 %RSD_r—2.2–7.4 %RSD_R—6.8–24	*J. AOAC Int.*, 86, 400, 2003.[62]
Infant formula, fortified milk/D_3	Saponification, ambient, overnight. Extract w/Hex, 2×. Evaporate the extract to dryness, redissolve in MeOH	XTera™ RP18 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:water (92:8) containing 2.5 mM HAC and NaOAC Flow rate—1.5 mL min^{-1}	EC	DL—48 ng 100 g^{-1} QL—156 ng 100 g^{-1} %RSD_r—4.1 %RSD_R—4.4	*Anal. Chim. Acta*, 543, 58, 2005.[97]
Fish/D_3	Cold saponification. Clean-up: SPE Chem Elut column and semiprep NP LC	Supelco C_{18} 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH containing 8.4 mM $HClO_4$ and 57 mM $NaClO_4$ Flow rate—1 mL min^{-1}	EC	%CV—1.5–2.7 %Recovery—70–102	*Eur. Food Res. Technol.*, 222, 403, 2006[98]
Pharmaceuticals					
Medical nutritional products/D_2, D_3	Saponification. Extract w/Et_2O. Add D_2 (IS). Evaporate, redissolve in Hex. Clean-up: Semiprep LC, Nucleosil 50-5, 5 µm	Hitachi Gel 3056 5 µm, 25 cm × 4 mm Mobile phase—isocratic MeOH:MeCN: 50% perchloric acid (970:30:1.2) containing 0.057 M Sodium perchlorate Flow rate—1.2 mL min^{-1}	Dual electrode EC redox mode 0.65 V and 0.20 V H_2/H^+—reference	DL (on-column)—200 pg %Recovery—97 RSD_R—3.6	*J. Chromatogr.*, 605, 215, 1992[99]
Pharmaceutical products/D_2, D_3	Add 5 mL EtOH and 5 mL 0.1 M orthophosphoric acid to 0.2 g ground tablet fine powder, vortex. Extract it w/10 mL Hex, evaporate the Hex extract to dryness, redissolve in mobile phase	Nucleosil ODS 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic 95% MeOH:0.05 M acetate buffer (pH 6.0) flow rate—2.0 mL min^{-1}	EC +1.3 V versus Ag/AgCl	—	*Analyst*, 117, 1441, 1992[100]

Continued

Table 2.4 (Continued)

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Pharmaceuticals (*Continued*)					
Nutritional supplements/D_2	Dissolve sample in 0.2 M K_2HPO_4 aqueous solution containing 1 mM EDTA·2Na·$2H_2O$ Clean-up: Bond Elut C_{18} cartridge	Inertsil ODS-2 5 μm, 15 cm × 4.6 mm Mobile phase—isocratic MeOH 100% Flow rate—0.8 mL min^{-1}	265 nm	DL (on-column)—0.1 ng %RSD—6.2 %Recovery—80	*J. Chromatogr. A*, 881, 189, 2000[101]
Pharmaceutical preparations/D_2, D_3	For D_2—extract sample w/Et_2O, 2×, centrifuge, evaporate combined extracts to dryness, redissolve in THF For D_3—dissolve sample in THF	Hicrom C_{18} 5 μm, 25 cm 4.6 mm Mobile phase—isocratic for D_2 w/MeOH:MeCN (40:60); gradient for D_3 w/MeOH–THF Flow rate—1 mL min^{-1}	265 nm	—	*J. Liq. Chrom. Rel. Technol.*, 24, 973, 2001[102]
LC-MS					
Standards/D_3, 25(OH)D_3, 24, 25-$(OH)_2D_3$, 1α,25-$(OH)_2$ D_3, 1α-(OH) D_3	—	Rosil C_8—DA 5 μm, 15 cm × 4.6 mm Mobile phase—isocratic MeCN:water (80:20) Postcolumn derivatization Total flow rate—1 mL min^{-1}	MS–TSP positive and negative ion mode w/SIM	DL 60–100 nM (positive mode) 50–70 nM (negative mode)	*Biol. Mass Spectrom.*, 22, 621, 1993[103]
Multivitamin tablets/D_2, D_3, 25(OH)D_3, 1α-(OH) D_3	Dissolve sample powder in water—EtOH (1:1), extract w/Hex, 3×. Evaporate combined extracts, redissolve in mobile phase	TSK-gel 80TM ODS 5 μm, 15 cm × 4.0 mm Mobile phase—isocratic MeOH:water (9:1) Flow rate—1 mL min^{-1}	MS-APCI positive ion mode w/SIM	LD (on-column)—0.4–1.0 ng %RSD—1.9–5.7 %Recovery—104.4	*Anal. Sci.*, 10, 457, 1994[104]
Standards/D_3, 3—fatty acid esters	—	YMC-Pack C_8 5 μm, 15 cm × 4.6 mm Mobile phase—isocratic MeOH containing 10 mM NH_4OAC Flow rate—1 mL min^{-1}	MS-APCI positive ion mode w/SIM	—	*J. Liq. Chrom. Rel. Technol.*, 22, 367, 1999[105]
Feeds					
Animal feeds, premixes/D_2, D_3	Saponification, ambient, overnight. Extract w/Hex, no IS	SupelcoSil LC-18 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:MeOH (90:10) Flow rate—1.8 mL min^{-1}	301 nm	QL—1 IU g^{-1}	*J. AOAC Int.*, 75, 812, 1992[106]

Fish meat/D_2, Pre-D_3	Saponification, extract w/Et_2O. Evaporate, redissolve in MeOH, no IS	HC ODS/PAH, 25 cm × 2.6 mm & LC-18 5 μm, 15 cm × 4.6 in series Mobile phase—isocratic MeCN:MeOH:water (74:18:8) Flow rate—1.5 mL min^{-1}	255 nm	—	*Animal Feed Sci. Tech.*, 47, 99, 1994[107]
Biologicals					
Plasma/D_3, 25(OH)D_3, 25(OH)D_2	Add ^{3}[H]-D_3 and ^{3}[H]-25 (25)D_3 (IS) for recovery calculation. Denature protein w/MeOH:IPA (90:10), extract w/Hex	SupelcoSil LC-18 5 μm, 25 cm × 4.6 mm Mobile phase—gradient MeOH–water–IPA Flow rate—2.3 mL min^{-1}	265 nm	DL—5 mol L^{-1} %Recovery—79.9 %CV—5.3–7.0	*Scand. J. Clin. Invest.*, 52, 177, 1992[108]
Plasma/D_3, 25(OH)D_3, 25(OH)D_2, 24, 25-$(OH)_2D_2$, 1,25-$(OH)_2$ D	Add THF and EtOAC, centrifuge. Remove EtOAC layer, reextract w/EtOAC. Evaporate, redissolve in IPA:Hex (20:80) Clean-up: Semiprep HPLC	For D_2, D_3—Nucleosil 5 C_{18}, 30 cm × 7.5 mm w/mobile phase—MeCN:MeOH (50:50) Flow rate—2 mL min^{-1} For 25(OH)D_3, D_2—Zorbax SIL, 25 cm × 4.6 mm w/mobile phase—Hex:IPA (97.5:2.5) Flow rate—1.3 mL min^{-1}	CPBA for 24, 25-$(OH)_2$ D RRA for 1,25-$(OH)_2$ D 265 nm for the rest	DL (on-column)—0.002–0.5 ng %CV—4.6–9.6	*Food Chem.*, 45, 215, 1992;[41] *J. Nutr. Sci. Vitaminol.*, 39, 101, 1993[109]
Plasma/25(OH)$D_2$3S	Add 25 (OH)-7-dehydrocholesterol (IS) in EtOH. Deproteinize w/0.4 M phosphate, pH 7.5 Clean-up: SPE	YMC-Pack ODS-AM, 5 μm, 15 cm × 4.6 mm at 40°C Mobile phase—isocratic MeCN:$NaClO_4$ (98:2) Flow rate—1 mL min^{-1}	265 nm	QL—5 ng mL^{-1} %Recovery—95–104 %RSD—1.4–3.2	*Biomed. Chromatogr.*, 9, 229, 1995[110]
Plasma/25(OH)D_3	Add ^{3}[H]-25-D_3 for recovery and 5Z-22-OH-24,25,26,27-tetranor vitamin D_3 (IS). Clean-up: Sep-Pak silica	Zorbax SIL, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic Hex:$CHCl_3$:MeOH (100:25:2) Flow rate—1.5 mL min^{-1}	265 nm	QL—19 ng mL^{-1}	*J. Chromatogr. B: Biomed. Appl.*, 672, 63, 1995[111]
Human plasma/1α,25-$(OH)_2$ D_3	Add ^{3}[H]-1,25 $(OH)_2D_3$, extract w/CH_2Cl_2:MeOH (1:2) Clean-up: Bond Elute NH_2 LiChrosphere Si 60	YMC-Pak ODS-AM 302 5 μm, 15 cm × 4.6 mm Mobile phase—gradient MeOH—water Flow rate—1 mL min^{-1}	DMEQ-TAD adducts Fluorescence Ex λ = 370 Em λ = 440	QL—1.2 pg mL^{-1} %Recovery—88–106	*J. Chromatogr. B*, 690, 15, 1997[112]

Continued

Table 2.4 (Continued)

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Biologicals (*Continued*)					
Human plasma/ 25 (OH)D, 24,25$(OH)_2$D	Extract diluted plasma (1 to 5 in water) w/MeOH and CH_2Dl_2, evaporate and dissolve in IPA: Hex (5.5:94.5) Clean-up: HPLC (Zorbax SIL)	Nucleosil 5C_{18}, 30 cm × 7.5 mm Mobile phase—isocratic MeOH:MeCN (5:95) w/0.025 M $HClO_4$ Flow rate—1.2 mL min^{-1}	ECD	DL—50 pg mL^{-1} %Recovery—81.5 %RSD_r—5.3–9.7 %RSD_R—6.3–9.7	*J. Pharm. Biomed. Anal.*, 15, 1497, 1997[113]
Plasma/D_3, 1,25-$(OH)_2$ D_3, 24,25 $(OH)_2$ D_3, 25 (OH) D_3	Extract sample w/IPA, 4×, evaporate and extract w/Hex, 4×. Evaporate to dryness. Clean-up: Aminopropyl minicolumn	Ultrabase C_{18} 5 µm, 25 cm × 4.6 mm Mobile phase—gradient MeCN–IPA–MeOH–phosphate buffer Flow rate—1.2 mL min^{-1}	Post column derivatization Fluorescence Ex λ = 355 Em λ = 482	DL—0.01 pg mL^{-1} %RSD—2.6–4.7	*Chromatographia*, 50, 399, 1999[114] & *Talanta*, 50, 57, 1999[115]
Human serum/D_3	Extract sample w/IPA, 4×, evaporate and extract w/Hex, 4×. Evaporate to dryness. Clean-up: Aminopropyl minicolumn	Ultrabase C_{18} 5 µm, 25 cm × 4.6 mm Mobile phase—gradient MeOH–water Flow rate—1.0 mL min^{-1}	265 nm	%RSD_r—1.9–4.8 %RSD_R—2.8–8.8	*Anal. Bioanal. Chem.*, 377, 287, 2003[116]
Plasma/D_3, 25(OH) D_3	Extract sample w/MeCN at ratio of 1:3 and 1:10 for normal animals and supplemented animals. Centrifuge, collect supernatant	RP C_{18} 5 µm, 15 cm × 4.5 mm Mobile phase—isocratic 100% MeCN Flow rate—2.0 mL min^{-1}	264 nm	%Recovery—90–100	*Int. J. Vitam. Nutr. Res.*, 73, 15, 2003[117]
Human plasma/ D_3, 25(OH) D_3	Extract sample w/2 mL EtOH: MeCN (2:1), evaporate supernatant to dryness, redissolve in MeOH:5 mM phosphate buffer (4:1), pH 6.5. Clean-up: BioTrap 500 C_{18}	C_{18} 5 µm, 25 cm × 4.6 mm Mobile phase—gradient MeCN–MeOH–phosphate buffer (5 mM, pH 6.5)—THF Flow rate—1.4 mL min^{-1}	265 nm	%CV_{Intra}—1.5–2.8 %CV_{Inter}—1.4–2.8 %Recovery—91–98	*Talanta*, 64, 1364, 2004[118]
LC-MS					
Plasma/D_2, D_3, 25$(OH)D_3$, 24, 25-$(OH)_2D_3$, 1α, 25-$(OH)_2$ D_3	Extract sample w/MeCN, mix the extract w/K_2HPO_4 buffer (pH 10.4) to 50% aqueous. Clean-up: Waters C_{18} Sep-Pak cartridge	Hypersil C_{18} 5 µm, 25 cm × 4.6 mm Mobile phase—gradient MeOH–water (w/0.4 M NH_4OAC, pH 6.5, added after column, 0.3 mL min^{-1}) Flow rate—0.9 mL min^{-1}	MS–TSP positive ion mode w/SIM	—	*Biomed. Chromatogr.*, 5, 153, 1991[119]

Human plasma/ 25(OH) D_3, 25(OH) D_2	Add MeCN and 25(OH)D_4(IS) to the sample, vortex, centrifuge and evaporate. Redissolve in EtOAC: water (2:1), collect EtOAC layer, extract water layer w/EtOAC again, evaporate combined EtOAC layer. Residue undergoes the derivatization process	YMC ODS H-80 4 μm, 15 cm × 4.6 mm Mobile phase—isocratic MeOH:water (9:1) for 25(OH) D_3 MeCN:water (5:1) for 25(OH) D_2 Flow rate—1 mL min^{-1}	MS/MS-APCI positive ion mode w/MRM	DL (on-column)—5–8 pg %RSD_r—3.2–4.0 %Recovery—92.9–109.8	*Biol. Pharm. Bull.*, 24, 738, 2001[120]
Urine/25 (OH)D_3, 23, 25 $(OH)_2$ D_3, 24, 25 $(OH)_2$ D_3, 25, 26 $(OH)_2$ D_3, etc.	Samples go through enzymatic digestion. Extract the solution w/ MeCN Clean-up: OASIS HLB cartridge. Evaporate the eluate and residue undergoes the derivatization process	YMC ODS H-80 4 μm, 15 cm × 4.6 mm Mobile phase—isocratic MeOH:water (13:7) or MeCN: water (2:3) Flow rate—1 mL min^{-1}	MS/MS-APCI positive ion mode w/MRM	—	*J. Pharm. Biomed. Anal.*, 29, 947, 2002[121]
Human plasma/ 25(OH)D_3	Add $^3[H]_3$-^{13}[C]-25(OH)-D_3 (IS), mix. Add 1 M NaOH, MeCN, hold at 4°C, 1 h. Centrifuge. Online SFE (Oasis HLB, 20 mm × 2.1 mm, 25 μm)	100 RP-18, end-capped, 12.5 cm × 4 mm, 5 μm Mobile phase—isocratic Water:MeOH (95:5) Flow rate—3 mL min^{-1}	MS/MS-APCI positive ion mode	%Recovery—91 ± 1.6	*Clin. Chem.*, 50, 1415, 2004[122]
Human plasma/ 25(OH)D_2, -D_3	Add [$^2H_6$]-25(OH)D_3 (IS) in EtOH, mix, centrifuge. SPE (Bond Elute, C_{18}). Evaporate, dissolve residue in MeOH	CAPSELL PAK C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:water (95:5) Flow rate—0.5 mL min^{-1}	MS/MS-APCI Positive ion mode w/MRM	%Recovery—2.2–5.2 %RSD_r—4.5–11.4 %RSD_R—2.5–9.9	*Anal. Chem.*, 77, 3001, 2005[123]
Human plasma/ 25(OH)D_2, -D_3	Add [$^2H_6$]-25(OH)D_3 (IS) in EtOH, mix, centrifuge. SPE (Bond Elute, C_{18}). Evaporate, dissolve residue in MeOH	CAPSELL PAK C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:water (95:5) Flow rate—0.5 mL min^{-1}	MS/MS-ESI Positive ion mode w/MRM	%CV—5.1–9.5	*Clin. Chem.*, 51, 1683, 2005[124]
Human plasma/ 25(OH)D_2, -D_3	Add deuterated Δ^9-tetra-hydrocanabinol (IS) in MeOH, mix. Extract w/*n*-heptane, evaporate *n*-heptane layer, redissolve in EtOH	xTerra C_{18}, 3.5 μm, 50 mm × 2.1 mm Mobile phase—isocratic MeOH containing 2 mm NH_4OAC L^{-1} and 0.1% formic acid Flow rate—100 μL min^{-1}	MS/MS-ESI Positive ion mode w/MRM	%CV < 11	*Am. J. Clin. Pathol.*, 125, 914, 2006[125]

species of importance in Germany. Procedures developed by Kurmann and Indyk[84] and Matilla and colleagues[90,93] are provided in Section 2.4.

Microwave-assisted[128] and supercritical fluid extraction (SFE)[129] has been used to extract ergosterol from mushrooms. Because of the significance of saponification to analysis of vitamin D in food, various saponification procedures are summarized in Table 2.5. Most procedures are completed at ambient temperatures with pyrogallol or ascorbic acid added as antioxidants. Care must be taken to limit thermal isomerization of vitamin D to previtamin D. Ball[127] discusses the problem of resolution of previtamin D from coeluting interfering compounds in both normal-phase and reversed-phase LC systems. Reflux temperatures lead to previtamin D formation and, if not resolved by chromatography, calculations must correct for the percentage conversion during extraction.[127] For this reason, internal standards (Section 2.3.2.3) are routinely used for food analysis and considered essential.

Extraction of serum does not require saponification; therefore, degradative conditions can be avoided. Initial procedures rely on protein denaturation and solvent partitioning. Commonly used procedures are summarized in Table 2.4. Jones and colleagues[48] classified solvent extractions into the following general approaches:

6. Total lipid extraction—methanol:chloroform:water (2:1:0.8), which is the Bligh and Dyer technique or ethanol:water (9:1)
7. Selective lipid extraction—ether, ethyl acetate:cyclohexane (1:1), hexane, dichloromethane, or hexane:isopropanol (1:2)

Jones and colleagues[48] point out that the Bligh and Dyer extraction using methanol and chloroform is highly efficient for extraction of hydroxylated metabolites. Also, dichloromethane can be substituted for chloroform to avoid the use of the carcinogen. Such extractions based on lipid extraction provide the initial procedural step in methods that rely upon CPBA, RRA, or RIA for quantitation of low-level metabolites.

Solid-phase chromatographic cleanup is an indispensable approach for both food and clinical sample analysis. Use of normal-phase silica and reversed-phase C_{18} cartridges are routine (Table 2.4).

2.3.3.2 *Chromatography parameters*

2.3.3.2.1 Supports and mobile phases. Both normal- and reversed-phase systems offer efficient resolution of vitamins D_2, D_3, the previtamins, and hydroxylated metabolites, if properly prepared extracts are available. Most procedures rely on silica or C_{18} supports. C_8 and cyano-columns are used less frequently. Chromatography systems for vitamin D_3 metabolites and vitamin D_3 in dairy products were reviewed by Luque de Castro and colleagues[50] and Perales and colleagues[52] respectively. More recent methods take advantage of small-bore columns that significantly reduce solvent usage and run time.[124,125] Masuda and colleagues[41] (Figure 2.3) effectively demonstrated the usefulness of both chromatography modes in an integrated analytical approach to serum analysis. For vitamins D_2 and D_3, preparative LC or Zorbax SIL was followed by analytical LC on Nucleosil 5C_{18}. 25(OH)D_2 and –D_3 and the dihydroxy metabolites were concentrated by preparative LC on Nucleosil 5C_{18} and analytical LC on Zorbax SIL. Vitamins D_2 and D_3, 25(OH)D_2 and –D_3 were detected by UV at 265 nm. 24,25$(OH)_2D_2$ and -D_3 were assayed by CPBA, and 1α,25$(OH)_2D_2$ and -D_3 by RRA. Recovery was determined through addition of [1α,2α(n)-^{3}H]-D_3, [23,24(n)-^{3}H]-25(OH)D_3, [23,24(n)-^{3}H]-24,25$(OH)_2D_3$, and [26,27-methyl-^{3}H]-1α,25$(OH)_2D_3$ in ethanol before lipid extraction by tetrahydrofuran (THF) and ethyl acetate.

2.3.2.2.2 Detection. Detection at wavelengths near the UV maximum for vitamin D of 265 nm is sensitive enough for most matrices for the detection of vitamins D_2 and D_3 and

Table 2.5 Saponification Parameters for the Analysis of Vitamin D

Matrix	Sample size	Hydrolysis conditions	Antioxidant	Extractant	Internal standard or %Recovery	Quantitation level	References
Fortified milk	50 mL	KOH Ambient overnight	Ethanolic pyrogallol	Diethyl ether: Hexane (50:1)	D_2—98%	1.08 µg mL^{-1}	*J. AOAC*, 60, 998, 1977[75]
Fish	20 g	Ethanolic KOH Ambient overnight	—	Diethyl ether: petroleum ether (1:1)	D_2 (IS) and 25(OH)D_2 (IS)	0.2 µg 100 g^{-1}	*J. Food Compos. Anal.*, 8, 232, 1995[89]
Baby foods	50 g	Ethanolic KOH 70°C, 30 min	Pyrogallol Nitrogen flush	Diethyl ether: petroleum ether (1:9)	D_2 (IS) or D_3 (IS) depending on product form	400 IU kg^{-1}	*Neth. Milk Dairy J.*, 48, 31, 1994[86]
Egg yolk	20 g	Ethanolic KOH Ambient overnight	Ascorbic acid	Diethyl ether: petroleum ether (1:1)	D_2 (IS) or 25(OH)D_2 (IS)	D_2—0.4 µg 100 g^{-1} 25(OH)D_2—0.87 µg 100 g^{-1}	*J. Food Compos. Anal.*, 5, 281, 1992;[73] 6, 250, 1993[88]
Mushrooms	1.0 g (dry)	Ethanolic KOH Ambient overnight	Ascorbic acid	Diethyl ether: petroleum ether (1:1)	D_3 (IS) or 25(OH)D_3 (IS)	2.8 µg 100 g^{-1}	*J. Agric. Food Chem.*, 42, 2449, 1994[90]
Feeds, premixes	Feed 1.2 g Premix 0.5 g	Ethanolic KOH Ambient overnight	Pyrogallol	Hexane	92%—premix 87%—feed	1.0 IU g^{-1}	*J. AOAC Int.*, 75, 812, 1992[106]
Milk, margarine, infant formula	Milk—15 mL Infant formula—10 mL Margarine—5 g	KOH Ambient, 18 h	Ethanolic pyrogallol	Hexane	D_2—96–99%	382 IU 100 g^{-1}	*J. AOAC*, 65, 624, 1982[76]
Fortified milk powder	10 g	Ethanolic KOH Ambient overnight	Ethanolic pyrogallol Nitrogen flush	Diethyl ether: petroleum ether (10:90)	>95%	30 IU 100 g^{-1}	*NZ J. Dairy Sci.*, 19, 19, 1984[77]
Milk, infant formula	Powder—4 g Infant formula—30 mL	Ethanolic KOH 75°C, 30 min	Ascorbic acid	Diethyl ether: petroleum ether (1:1)	96–97%	348 IU L^{-1}	*J. AOAC*, 68, 177, 1985[67]
Milk powder, infant powder	10 g	Ethanolic KOH Reflux, 45 min	Sodium ascorbate	Benzene	$[^3H]D_3$	5 IU g^{-1}	*J. Agric. Food Chem.*, 34, 264, 1986[79]

Continued

Table 2.5 (Continued)

Matrix	Sample size	Hydrolysis conditions	Antioxidant	Extractant	Internal standard or %Recovery	Quantitation level	References
Fortified milk, infant formula, milk powder	Powder—20 g Liquid—12 g	Ethanolic KOH Ambient overnight	Sodium ascorbate Nitrogen flush	1-Chlorobutane	D_3—95%	19.3 IU 100 g^{-1} (powder)	*Neth. Milk Dairy J.*, 42, 423, 1988[81]
Infant formula, enteral nutritionals	15 mL	Ethanolic KOH 60°C, 30 min	—	Hexane (IS) depending on product form	D_2 (IS) or D_3	136 IU qt^{-1}	*J. AOAC Int.*, 75, 566, 1992;[68] 79, 73, 1996[69]
Fortified milk	15 mL	Ethanolic KOH 60°C, 30 min	—	Hexane	D_2 (IS) 81–96%	1.6 μg L^{-1}	*J. AOAC Int.*, 77, 1047, 1994[83]
Fish	2–10 g	Ethanolic KOH Ambient overnight	Ethanolic pyrogallol Nitrogen flush	SPE Chem Elut	D_2 (IS) Pre D_2 (IS) 70–102%	50 ng mL^{-1}	*Eur. Food Res. Technol.*, 222, 403, 2006[98]

$25(OH)D_2$ and $-D_3$, provided proper extract purification steps are included in the sample preparation protocol. UV detection is not sensitive and specific enough for the quantitation of dihydroxy metabolites in serum and tissue at the normal physiological levels of less than 100 pg per mL. Requirements for the clinical assay of dihydroxy metabolites were the impetus for the development of the CPBA, RRA, and RIA procedures more than three decades ago (Section 2.3).

Since vitamin D does not fluoresce, this powerful detection mode has not been available for routine vitamin D assay. A fluorometric method for analysis of $1\alpha,25(OH)_2D_3$ from plasma entailed the fluorescence labeling of the vitamin D metabolite with 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydro-quinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD).[112] The adducts were fluorometrically detected at Ex $\lambda = 370$ and Em $\lambda = 440$. The limit of detection for $1\alpha,25(OH)_2D_3$ was 5 pg and CV% values were around 10%. Recoveries were low, but values corrected for recovery compared closely to RRA determined values. In its present state, the new procedure is more tedious and time consuming compared to RRA methodology. However, it is the first successful application of fluorescence detection to $1\alpha,25(OH)D_3$ in plasma.

Electrochemical detection, while used infrequently for vitamin D quantitation, was the detection mode in recent research quantifying vitamin D_3 in infant formula and fortified milk[97] and fish.[98] Perales and colleagues[97] optimized the detector by varying the applied potential in 50 mV steps to increase analyte signal and monitor background signal. Using this process, an optimal working potential of +1450 mV was selected for analysis of vitamin D_3. Response linearity ranged from 0.03 to 0.7 µg D_3 mL^{-1} with DL and QL of 48 ng 100 g^{-1} and 156 ng 100 g^{-1}, respectively.

Liquid chromatography coupled with mass spectrometry (LC-MS) is rapidly becoming the method of choice for analysis of $25(OH)D_3$ in serum. Sensitive and highly selective methods are now available for the clinical laboratory. These methods use electrospray ionization (ESI) [124] and, more commonly, atmospheric pressure chemical ionization (APCI).[120–123,125] Procedures published from 2001 to 2006 use tandem mass spectrometry (LC-MS/MS). LC-MS methodology has not been used extensively for food analysis. Heudi and colleagues[130] developed an LC-MS procedure for the simultaneous assay of vitamins A, D_3, and E and fortified infant formula, which is discussed in Chapter 14.

2.3.3.3 Internal standards

Owing to the complexity of sample treatments for vitamin D analysis, internal standards are required to compensate for extraction losses. Vitamin D_2 can be used as the internal standard for vitamin D_3 procedures or vice versa, provided the internal standard is proven to be absent from the matrix. Likewise, for $25(OH)D_2$ or $-D_3$, the hydroxylated metabolite not present in the matrix can be conveniently used as the internal standard. Deuterated metabolites are routinely added to clinical samples for recovery calculations pertinent to the assay of low-level metabolites.

2.4 Method protocols

Vitamin D_3 in Fluid and Spray Dried Milk

Food Chem., 50, 75, 1994[84] and 57, 283, 1996.[85]

Principle

Samples were saponified, extracted with hexane:diethyl ether (9:1), and fractionated by SPE silica and semipreparative LC on Resolve silica Rad-Pak columns. The purified extracts were chromatographed on Resolve C_{18} with UV detection of vitamin D at 265 nm. Vitamin D_2 was added before saponification as the internal standard.

Chemicals

- Ethanol
- Potassium hydroxide
- Hexane
- Anhydrous diethyl ether
- Pyrogallol
- Methanol
- Ethyl acetate
- Tetrahydrofuran
- Isopropyl alcohol
- Anhydrous sodium sulfate
- Vitamins D_2 and D_3 USP standards

Apparatus

- Liquid chromatograph
- UV detector
- Sep-Pak silica cartridges
- Rotary evaporator

Sample Preparation

- Add 250 mL of 30% ethanolic KOH containing 1% pyrogallol to 100 mL fluid milk, vitamin D_2 internal standard, 1 mL
- Flush with N_2, saponify 16 h at ambient temperature
- Transfer to separatory funnel with 100 mL water wash
- Extract 2× with 500 mL hexane:diethyl ether (9:1)
- Wash combined organic phase with water until neutral
- Filter extract through 25 g Na_2SO_4
- Evaporate to near dryness
- Dissolve residue in 1 mL hexane

SPE Purification

- Pass 10 mL hexane through Sep-Pak silica cartridge
- Load sample extract
- Wash cartridge with 3 mL hexane:ethyl acetate (90:10)
- Elute vitamin fraction with 5 mL hexane:ethyl acetate (80:20)

Semipreparative HPLC Fractionation

- Inject entire vitamin D fraction on 8 × 10 Radial Compression Module containing Resolve silica Rad-Pak (5 µm) column
- Mobile phase—hexane:isopropyl alcohol (99:1), isocratic, 1 mL min^{-1}
- Monitor eluate at 265 nm
- Collect vitamin D fraction between 2 min before and 2 min after established peak retention time
- Evaporate to dryness under N_2
- Dissolve immediately in 100 mL methanol

Analytical Chromatography Parameters*

Column	Resolve C_{18} (5 mm) Rad-Pak 8 × 10 Radical Compression Module
Mobile phase	Methanol:THF:water (93:2:5), isocratic
Flow rate	1 mL min^{-1}

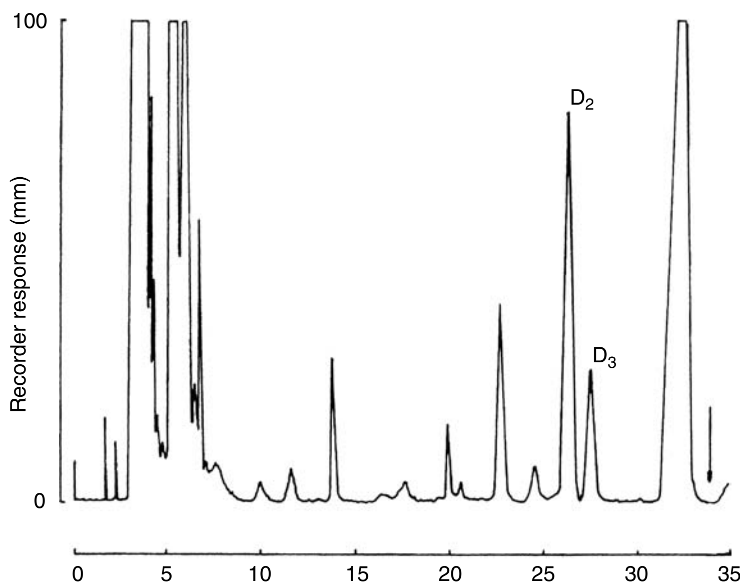


Figure 2.4 Chromatogram of endogenous vitamin D3 in bovine milk by the method of Kurmann and Indyk. (Reproduced with permission from Kurmann, A. and Indyk, H., *Food Chem.*, 50, 75, 1994.)

Temperature	Ambient
Detection	265 nm and PDA
Calculation	Internal standard, peak height

*See Figure 2.4.

Vitamin D Contents in Edible Mushrooms

J. Agric. Food Chem., 42, 2449, 1994[90] and 57, 95, 1996.[93]

Principle

Saponified samples are extracted with diethyl ether:petroleum ether (1:1), purified by SPE on silica and fractioned by semipreparative LC on mPorasil or mPorsail followed by reversed-phase chromatography on Vydac 201TP54. Analytical chromatography used either normal-phase or reversed-phase systems with detection at 264 nm.

Chemicals

- Vitamin D_2, D_3, 25(OH)D_3, and 25(OH)D_2 standards
- Ethanol
- Potassium hydroxide
- Diethyl ether
- Methanol
- Hexane
- Isopropanol

Apparatus

- Liquid chromatograph
- UV detector, diode array detector
- Rotary evaporator
- Vacuum oven

Procedure

Sample Extraction

- Add internal standards—vitamin D_3 for vitamin D_2 and 25(OH)D_3 for 25(OD)D_2 or vice versa. External spiking used if sample contains both D_2 and D_3 for recovery corrections
- Saponify overnight—ambient
- Extract with petroleum ether:diethyl ether (1:1)
- Wash extract with water until neutral
- Evaporate with Rotavapor
- Dissolve in 1.5 mL hexane

Sep-Pak Purification (Margarine, Egg Yolk)

- Dilute extract with 10 mL hexane and add to Mega Bond Elut silia column previously washed with 20 mL hexane
- Wash with 20 mL hexane
- Wash with 50 mL of 0.5% isopropanol in hexane
- Elute vitamin D fraction with 35 mL of 0.5% isopropanol in hexane
- Wash column with 50 mL of 0.5% isopropanol in hexane
- Elute 25(OH)D_2 and -D_3 with 40 mL of 6% isopropanol in hexane
- Evaporate 25(OH)D fraction to dryness and redissolve in 1.5 mL hexane
- Filter, 0.45 mm

Semipreparative HPLC Fractionation

- Inject 1 mL extract on µPorasil column
- Gradient elution

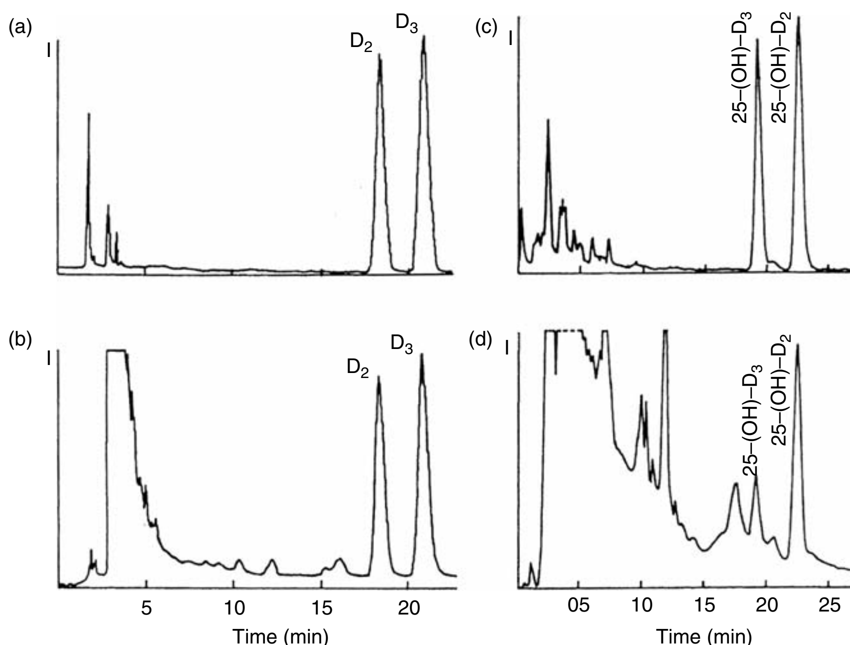


Figure 2.5 Chromatograms of vitamin D_2 and vitamin D_3 standards (a), vitamin D_2 (IS) and vitamin D_3 in Pike (b), 25(OH)D_2 and -D_3 standards (c), and 25(OH)D_3 in whitefish (d). (Reproduced from Matilla, P. H., Piironen, V. I., Uusi-Rauva, E. J., and Koivistoinen, P. E., *Food Chem.*, 57, 95, 1996. With permission.)

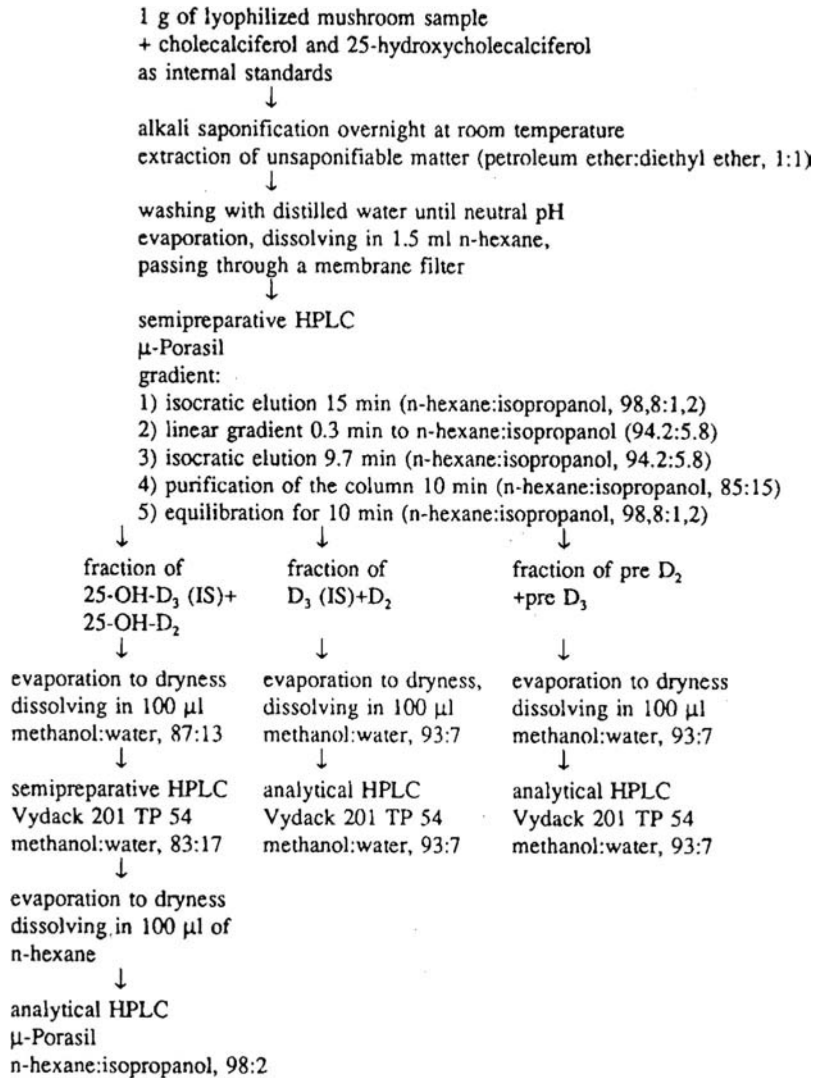


Figure 2.6 Analysis protocol for the analysis of vitamin D_2, $25(OH)D_2$, previtamin D_2 mushrooms. (Reproduced from Mattila, P. H., Piironen, V. I., Uusi-Rauva, E. J., and Koivistoinen, P. E., *J. Agric. Food Chem.*, 42, 2449, 1994. With permission.)

- Isocratic, 15 min, hexane:isopropanol (98.8:1.2)
- Linear gradient, 0.3 min to hexane:isopropanol (94.2:5.8)
- Isocratic, 9.7 min, hexane:isopropanol (94.2:5.8)
- Column purification, 10 min, hexane:isopropanol (85:15)
- Equilibration, 10 min, hexane:isopropanol (98.8:1.2)

Notes

- Previtamin D_2 and D_3, and D_3 elute as one peak at 8 min and 12 min, respectively
- $25(OH)D_2$ and $-D_3$ resolve between 20 and 25 min
- Collection times—1.5 min before and after retention times of previtamins and D_2 and D_3; 2 min before $25(OH)D_2$ and 2 min after $25(OH)D_3$ elution

Analytical Protocol
See Figures 2.5 and 2.6.

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chapter three

Vitamin E: tocopherols and tocotrienols

3.1 Review

The existence of vitamin E was first indicated through reproductive studies with animals in the early part of this century. The vitamin was characterized as a fat-soluble nutritional factor, "Factor X," necessary for reproductive functions and the prevention of fetal death by Evans and Bishop in 1922.[1] It then became known as the antisterility factor with the designation of vitamin E, since its discovery closely followed vitamin D. Vitamin E (α-tocopherol, α-T) was isolated from wheat germ oil by Evans' research group (1936).[2] These researchers called vitamin E "tocopherol" from the Greek terms pherein ("carry") and tocos ("to birth"), owing to its essentiality for rats to bear the young.[3] The 'ol' suffix denotes that the compound is an alcohol.[4]

The early history of vitamin E is further defined by the isolation of β- and γ-tocopherol (β-, γ-T) from vegetable oil in 1937,[5] determination of the structure of α-T in 1938,[6,7] synthesis of α-T in 1938,[8] recognition of the antioxidant activity of the tocopherols,[9] recognition that α-T was the most effective tocopherol in prevention of vitamin E deficiency,[5] isolation of δ-tocopherol (δ-T) from soybean oil in 1947,[10] and identification of the four naturally occurring tocotrienols (α-T3, β-T3, γ-T3, δ-T3).[11,12] Many excellent reviews cover all aspects of vitamin E knowledge. Each review has many outstanding qualities; anyone interested in the early history of vitamin E should read the publication of the Symposium on Vitamin E and Metabolism in honor of Professor H. M. Evans.[13]

Deficiency occurs infrequently in humans and is almost always due to factors other than dietary insufficiency. Vitamin E deficiency can occur through various conditions that affect absorption of fat and/or vitamin E. Malabsorption results from pancreatic and liver abnormalities that lower fat absorption, abnormalities of the intestinal cells, length of the intestine, and defects in the synthesis or assembly of the chylomicrons.[14] Genetic abnormalities in lipoprotein metabolism that produce low to nondetectable circulating chylomicrons, very-low-density lipoprotein (VLDL), and low-density lipoprotein (LDL) levels affect both absorption and plasma transport of vitamin E.[15] Genetic defects in the α-tocopherol transfer protein gene are associated with the syndrome "ataxia with vitamin E deficiency (AVED)" characterized by neurologic abnormalities.[16]

Symptoms of vitamin E deficiency are difficult to categorize owing to varying effects among species and the fact that vitamin E deficiency in the human is primarily due to fat malabsorption syndromes or genetic abnormalities. At the cellular level, vitamin E deficiency produces increased oxidation of cellular membranes.[17] Related events might include decreased energy production by mitochondria, DNA mutation, and changes in plasma membrane transport mechanisms. Olson and Munson[14] give the following disorders associated

with vitamin E deficiency: reproduction disorders, abnormalities of muscle, liver, bone marrow, brain function, and defective embryogenesis diathesis (capillary permeability disorder). Skeletal muscle dystrophy is species specific and involves cardiomyopathy, which is severe in ruminants, mild in rabbits, and nonexistent in primates. The variable occurrence by species of vitamin E deficiency symptoms was noted as an impediment to in-depth understanding of the vitamin function at the cellular and molecular level.[14] Primary symptoms in humans include mild hemolytic anemia characterized by increased erythrocyte hemolysis and spinocerebellar diseases, usually in children suffering from fat malabsorption syndrome.[14] Clinical status of vitamin E in the human is difficult to assess owing to extremely slow depletion of body stores. Biochemical indices include serum, erythrocyte, platelet, and tissue tocopherol levels; however, none are effective status indices.[17] α-T levels in serum <11.6 μmol L^{-1} in adults is an indication of a biochemical vitamin E deficiency but does not always indicate a clinical deficiency.[17,18] The Institute of Medicine, *Panel on Dietary Antioxidants and Related Compounds*,[23] considered hydrogen peroxide-induced hemolysis as the best biomarker used in conjunction with plasma α-T concentrations to estimate adult human requirements for α-T.

Abetalipoproteinemia is an autosomal recessive genetic disorder that leads to mutations in the microsomal triglyceride transfer protein.[19–22] The disease is associated with ataxia and impaired absorption of lipids, vitamin E, and other fat-soluble vitamins except vitamin D, since the triglyceride transfer protein participates in the intracellular transport of lipids and other fat-soluble substances. Deficiency of vitamins E, A, and K results in clinical symptoms associated with abetalipoproteinemia. The microsomal triglyceride transfer protein is completely absent from the intestines of abetalipoproteinemia patients.[22] Symptoms include steatorrhea with fat-engorged enterocytes, absence of apolipoprotein B in the plasma, and absence of intestinal staining for apolipoprotein B in the intestine.[20] Neurological symptoms including reflex changes, dyspraxia, and abnormal movements have been observed.[20]

Deficiency results from genetic abnormalities in production of the α-TTP, fat malabsorption syndromes, and protein-energy malnutrition.[23] Fat malabsorption can be related to pancreatic and liver abnormalities that lower fat absorption, abnormalities of the intestinal cells, length of the intestine, and defects in the synthesis or assembly of the chylomicrons.[19] Genetic abnormalities in lipoprotein metabolism can produce low levels of chylomicrons, VLDLs, and LDL that affect absorption and transport of vitamin E.[19]

Friedreich's ataxia is an autosomal recessive disease characterized by cerebellar ataxia, dysarthria, sensory loss in the lower limbs, and other neurological symptoms.[24,25] Early studies on Friedreich's ataxia identified a variant form characterized by normal fat absorption and very low levels of plasma vitamin E. Neurological symptoms were considered to be due to vitamin E deficiency.[24,25] Homozygosity mapping showed that Friedreich's ataxia is characterized by defects at chromosome 9;[25] whereas, the variant showed defects at chromosome 8.[26] With the specific differences noted at the chromosomal level, the newly recognized genetic defect was termed *familial isolated vitamin E deficiency*[26] or *ataxia with vitamin E deficiency* (AVED). AVED leads to an impaired ability to incorporate *RRR*-α-tocopherol (*RRR*-α-T) into VLDL.[27] Therefore, AVED was attributed to a defect in the α-TTP gene.[27] The primary cause of neurodegenerative symptoms in AVED patients is now known to be vitamin E deficiency due to the absence of a functioning α-TTP[28,29] with inefficient transfer of *RRR*-α-T from the liver and lack of recycling of plasma *RRR*-α-T. Clinical symptoms include many neurological problems stemming from peripheral neuropathy with degeneration of the large-caliber axons in the sensory neurons.[23] Common symptoms are ataxia, muscle weakness and hypertrophy, neurological abnormalities, reproductive disorders, and abnormalities of the liver, bone marrow, and brain.[14] At the cellular level, increased oxidation can

occur as a result of increased oxidative stress. The progression of vitamin E deficiency symptoms has been described as follows: hyporeflexia, ataxia, limitation in upward gaze, cross-eye, muscle weakness, visual field constriction, and centrocecal scotoma (dark spots in the eye).[20,30,31]

The preferential incorporation of α-T into nascent VLDL in the liver is accomplished by action of the α-TTP, which has been identified, isolated, and characterized from rat and human liver cytosol.[32–36] *In vitro*, the purified α-TPP transfers α-T between liposomes and microsomes.[33,37] The relative affinity of α-TTP was greatest for *RRR*-α-T when compared to other tocopherols, tocopheryl esters, and α-tocotrienol (α-T3). Calculated on the basis of degree of competition with *RRR*-α-T, the relative affinities were *RRR*-α-T = 100, *RRR*-β-T = 38, *RRR*-γ-T = 9, *RRR*-δ-T = 2, α-tocopheryl acetate = 2, α-tocopheryl quinone = 2, *SRR*-α-T = 11, and α-T3 = 12.[37]

α-TTP can discriminate between *RRR*-α-T and other forms of vitamin E, most likely on the basis of the number and position of the methyl groups on the chromanol ring[38] and the stereoiosomerism at the two carbon of the chromanol ring of α-T. Hosomi et al.[37] concluded that the biological activity of various forms of vitamin E is dependent upon tissue delivery and that their affinities for α-TPP limit secretion into lipoproteins and ultimate delivery to tissues. Affinity for the α-TTP was, therefore, proposed as a major determinant of biological activity.

Human deficiency is generally not due to dietary insufficiency. Vitamin E is a plant product, widely distributed at good concentrations in plant oils. The contribution of vitamin E activity by oils and fats to the human diet is highly significant. This fact is apparent from data derived from U.S. consumption surveys. Data from the second National Health and Nutrition Examination Survey (NHANES II) showed that fats and oils accounted for over 20% of the vitamin E in the U.S. diet. Data collected from the Continuing Survey of Food Intakes by Individuals (CSFII, 1994) indicated that salad and cooking oils, margarine, salad dressings, mayonnaise, and shortening provide approximately 27% of the vitamin E in the U.S. diet.[39] The role of low-fat margarine and spreads is not clearly indicated by this data. Increasing use of low-fat products will impact the order of available sources. Low-fat margarines are not normally fortified with vitamin E in the United States, and vitamin E content decreases as fat content decreases. Low-fat mayonnaise-type products usually contain added α-tocopheryl acetate. Since soybean oil is the primary oil used in these products in the United States, its role as a major vitamin E source is apparent. Worldwide, the contribution of palm oil to oil supplies and, therefore, as a vitamin E source is increasing.[41]

Data taken from the United States Department of Agriculture, *National Nutrient Database for Standard Reference, Release 19*[42] is presented in Table 3.1, ranking common food sources on the basis of α-T (mg 100 g^{-1}) content. This tabulation shows that vegetable oils and various nuts are concentrated food sources of α-T when compared to other foods. A recent compilation of vitamin E content on the basis of literature studies was published by Eitenmiller and Lee.[43] This compilation covers research articles appearing in the literature from 1970 to 2002. Vitamin E analyses after 1980 were exclusively completed by liquid chromatography (LC). In 2006, Chun et al.[44] detailed the tocopherol and tocotrienol content of fruit and vegetables common to the U.S. marketplace. Data of Lee and Lee[45] detailed vitamin E sources in the Korean diet.

When establishing the Dietary Reference Intake (DRI) values for vitamin E, the panel on antioxidants and related compounds decided that human requirements should only be based on the *2R-isomers* of α-T (*RRR-*, *RSR-*, *RRS-*, *RSS-*).[23] This decision stems from the accumulated evidence of the strong selectivity of α-TTP for the 2-*R* isomers in the liver, which leads to preferential secretion of the 2-*R* isomers into nascent VLDL. Other forms of vitamin E (2*S*-isomers of α-T, β-T, γ-T, δ-T, and tocotrienols) are primarily secreted into the bile and excreted. The selection process is not 100% effective; however, only small amounts of other dietary and supplemental vitamin E forms are delivered to the cells. For adults,

Table 3.1 α-Tocopherol Content of Various Foods[a]

Description	NDB no	mg 100 g^{-1}	Description	NDB no.	mg 100 g^{-1}
Oil, wheat germ	04038	149.4	Snacks, tortilla chips, plain, white corn	19056	3.5
Cereals ready-to-eat, general mills, whole grain total	08077	45.0	Turnip greens, frozen, cooked, boiled, drained, without salt	11575	2.7
Cereals ready-to-eat, general mills, total corn flakes	08246	45.0	Tomato products, canned, sauce	11549	2.1
Oil, vegetable, sunflower, linoleic, (~65%)	04506	41.1	Fish, sardine, Atlantic, canned in oil, drained solids with bone	15088	2.1
Oil, vegetable, sunflower, high oleic (70% and over)	04584	41.1	Spinach, raw	11457	2.0
Oil, vegetable, cottonseed, salad or cooking	04502	35.3	Nuts, pistachio nuts, dry roasted, with salt added	12652	1.9
Oil, vegetable safflower, salad or cooking, oleic, over 70% (primary safflower oil of commerce)	04511	34.1	Spinach, canned, drained solids	11461	1.9
Seeds, sunflower seed kernels, dry roasted, with salt added	12537	26.1	Doughnuts, cake-type, plain (includes unsugared, old-fashioned)	18248	1.9
Nuts, almonds	12061	25.9	Fish, roughy, orange, cooked, dry heat	15232	1.9
Vegetable oil, canola	04582	17.1	Fast foods, biscuit, with egg and sausage	21005	1.6
Oil, vegetable, palm	04055	15.9	Peppers, sweet, red, raw	11821	1.6
Oil, peanut, salad or cooking	04042	15.7	Nuts, pecans	12142	1.4
Cereals ready-to-eat, Kellogg, Kellogg's Special K	08067	15.3	Soy milk, fluid	16120	1.4
Nuts, hazelnuts or filberts	12120	15.0	Broccoli, frozen, chopped, cooked, boiled, drained, without salt	11093	1.3
Oil, olive, salad or cooking	04053	14.4	Egg, whole, cooked, fried	01128	1.2
Oil, vegetable, corn, industrial and retail, all purpose salad or cooking	04518	14.3	Blackberries, raw	09042	1.2
Oil, soybean, salad or cooking (hydrogenated) and cottonseed	04543	12.1	Fast foods, chicken, breaded and fried, boneless pieces, plain	21229	1.1

Nuts, mixed nuts, dry roasted, with peanuts, with salt added	12635	10.9	Oat bran, raw	20033	1.0
Peanuts, all types, dry-roasted, without salt	16390	10.7	Shortening, household, lard and vegetable oil	04544	1.0
Nuts, pine nuts, dried	12147	9.4	Sweet potato, canned, vacuum pack	11512	1.0
Snacks, potato chips, plain, unsalted	19811	9.1	Egg, whole, raw, fresh	01123	1.0
Peanut butter, smooth style, with salt	16098	9.0	Beans, pinto, mature seeds, cooked, boiled, without salt	16043	0.9
Oil, soybean, salad or cooking (hydrogenated)	04034	8.1	Fish, tuna, light, canned in oil, drained solids	15119	0.9
Salad dressing, french, home recipe	04133	8.0	Fast food, Pizza Chain, 14″ pizza, pepperoni topping, regular crust	21302	0.9
Peanuts, all types, dry-roasted, with salt	16090	7.8	Fish, tuna, white, canned in water, drained solids	15126	0.9
Snacks, potato chips, plain, salted	19411	6.7	Wheat flour, whole-grain	20080	0.8
Nuts, brazilnuts, dried, unblanched	12078	5.7	Fast foods, potato, french fried in vegetable oil	21138	0.8
Snacks, potato chips, reduced fat	19422	5.5	Fast foods, cheeseburger, regular, double patty, plain	21092	0.8
Salad dressing, mayonnaise, soybean oil, with salt	04025	5.2	Peaches, raw	09236	0.7
Salad dressing, french dressing, commercial, regular	04120	5.0	Nuts, walnuts, english	12155	0.7
Margarine, regular, tub, composite, 80% fat, with salt	04611	5.0	Nuts, macadamia nuts, raw	12131	0.5
Candies, milk chocolate, with almonds	19132	4.5	Tomatoes, red, ripe, raw, year round average	11529	0.5
Snacks, corn-based, extruded, puffs or twists, cheese-flavor	19008	4.2	Nuts, chestnuts, european, roasted	12167	0.5
Salad dressing, thousand island, commercial, regular	04017	4.0	Waterchestnuts, chinese, canned, solids and liquids	11590	0.5
Margarine-butter blend, 60% corn oil margarine and 40% butter	04585	3.9	Milk, whole, 3.25% milkfat	01077	0.1

[a] Data from USDA National Nutrient Database for Standard Reference, Release 19 [42].

Table 3.2 Dietary Reference Intakes and Tolerable Upper Intake Levels for Vitamin E[a]

Life stage	DRI (mg d^{-1})	UL (mg d^{-1})
Infants (months)		
0–6	4	[b]
7–12	6	[b]
Children (years)		
1–3	**6**	200 (465 µmol)
4–8	**7**	300 (698 µmol)
Males (years)		
9–13	**11**	600 (1395 µmol)
14–18	**15**	800 (1860 µmol)
19–30	**15**	1000 (2326 µmol)
31–50	**15**	1000 (2326 µmol)
51–70	**15**	1000 (2326 µmol)
>70	**15**	1000 (2326 µmol)
Females (years)		
9–13	**11**	600 (1395 µmol)
14–18	**15**	800 (1860 µmol)
19–30	**15**	1000 (2326 µmol)
31–50	**15**	1000 (2326 µmol)
51–70	**15**	1000 (2326 µmol)
>70	**15**	1000 (2326 µmol)
Pregnancy (years)		
≤18	**15**	800 (1860 µmol)
19–30	**15**	1000 (2326 µmol)
31–50	**15**	1000 (2326 µmol)
Lactation (years)		
≤18	**15**	800 (1860 µmol)
19–30	**15**	1000 (2326 µmol)
31–50	**15**	1000 (2326 µmol)

[a] Bold type: Recommended dietary allowance; ordinary type: adequate intake (AI).

[b] Not possible to establish; source of intake should be formula and food only.

Source: Institute of Medicine, Food and Nutrition Board, *Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids*, National Academy of Sciences Press, Washington, D.C., 2000, Chap. 6

Estimated Average Requirement (EAR) and Reference Daily Intake (RDA) are 12 mg and 15 mg of α-T per day, respectively (Table 3.2). The upper intake level (UL) value includes all forms of α-T from supplemental intake of all-*rac*-α-T. The UL is 1000 mg d^{-1}.

Vitamin E and other antioxidant food components have, indeed, captured the interest of the world's consumers. Antioxidant components of the diet include vitamin E, ascorbic acid, carotenoids, selenium, and others such as the flavonoids.[41] Oxidative stress damage to the human includes the onset of disease states including:

1. Cancer—through initiation of carcinogenesis, promotion of tumor development mutagenesis, and stimulation of cell division
2. Cardiovascular disease—through oxidation of blood lipoproteins and the development of atherosclerosis, and oxidative damage to tissues during heart attack and stroke
3. Cataracts—through oxidative damage to the lens of the eye[46]

An accepted fact is that oxidative damage at the cellular level is significant to the onset of chronic disease. Vitamin E is the primary fat-soluble antioxidant in mammalian systems, and a balanced diet providing sufficient vitamin E and other plant origin antioxidants is desirable for optimal health and, possibly, provides for increased longevity with increased well-being. Direct benefits of supplemental vitamin E are not well defined and use of supplements to provide increased intake of vitamin E was not supported by the Panel on Dietary Antioxidants and Related Compounds.[23]

In earlier discussions, Block[46] stressed that new knowledge on disease-preventing effects of micronutrients will lead to new views on supplementation to close "the gap between the amounts of antioxidant nutrients found in typical diets and higher levels needed for optimal protection against chronic diseases."

Evidence indicates that antioxidant components of the diet have beneficial effects in prevention of various cancers, cardiovascular disease, and cataracts.[47,48] Studies indicate difficulty in differentiating the specific effects of vitamin E from other dietary antioxidants. Strong evidence exists that the antioxidant nutrients prevent development of cardiovascular disease. Kritchevsky[49] reviewed the role of vitamin E, vitamin C, and β-carotene in decreasing the susceptibility of LDLs to oxidation. Oxidized LDL is viewed as an initiator of atherosclerosis. Vitamin E was indicated as the most effective antioxidant in LDL, but the total antioxidant effect is a combined effect of all available antioxidants. Large epidemiological studies published as the Health Professional Studies involved 87,245 female nurses and 39,910 male health professionals and provided evidence of an association between a high intake of vitamin E and a lower risk of coronary heart disease in women and men. However, the studies could not prove cause-and-effect relationships.[50,51]

Generally, epidemiological studies indicate that diets rich in fruits and vegetables are associated with reduced risks for several cancers.[48,52] Again, it is difficult to ascertain specific effects for vitamin E. In a large nutrient intervention trial in Linxian, China, β-carotene, vitamin E, and selenium supplementation of the adult diet reduced stomach cancer risk in the population. No significant protective effects were present for other combinations of supplements.[53]

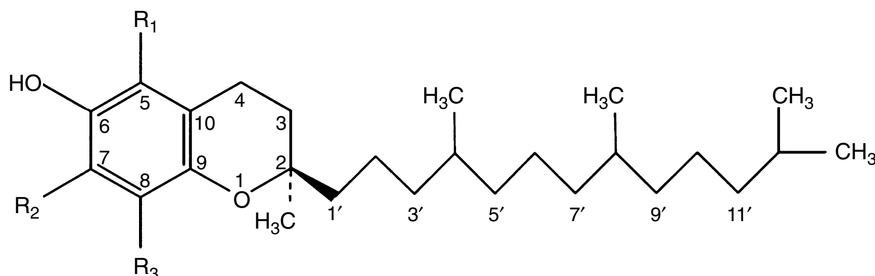
Cataracts are theorized to develop by photochemical generation of superoxide in the intraocular chambers of the eye.[48] Several studies have shown that supplementation with antioxidant vitamins and high dietary intakes are associated with lower cataract risk.[46] Likewise, low blood levels of vitamin E and β-carotene have been linked to increased risks.[23]

3.2 Properties

3.2.1 Chemistry

3.2.1.1 General properties

Vitamin E is the collective term for fat-soluble 6-hydroxychroman compounds that exhibit the biological activity of α-tocopherol measured by the rat resorption-gestation assay (Figure 3.1). Tocol (the parent compound for the vitamin E family) (Figure 3.1) is 2-methyl-2 (4′,8′,12′-trimethyltridecyl)-chroman-6-ol. Accepted nomenclature has been set by the International Union of Pure and Applied Chemistry—International Union of Biochemistry (IUPAC-IUB) Joint Commission on Nomenclature.[55–57] At present, eight naturally occurring homologs are included in the vitamin E family. The eight vitamin E forms are α-, β-, γ-, and δ-tocopherol characterized by a saturated side chain consisting of three isoprenoid units, and the corresponding unsaturated tocotrienols (α-, β-, γ-, and δ-). The tocotrienols have double bonds at the 3, 7, and 11 positions of the isoprenoid side chain. Structural interrelationships are shown in Figure 3.1. The tocopherol and tocotrienol homologs vary structurally

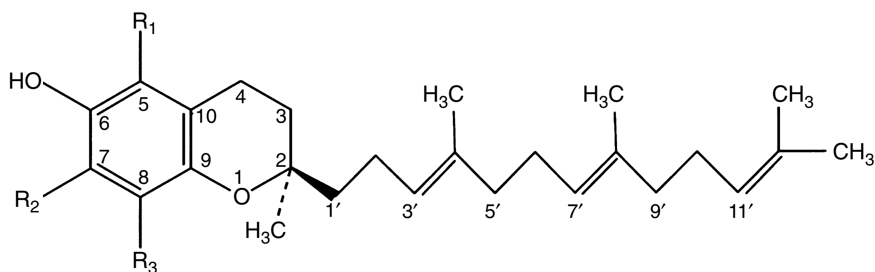


Compound I

Tocopherols

Trivial Name	Chemical Name	Abbreviation	Ring Position R[1]	R[2]	R[3]
Tocol	a	-	H	H	H
α-Tocopherol	5,7,8-Trimethyltocol	α-T	CH_3	CH_3	CH_3
β-Tocopherol	5,8-Dimethyltocol	β-T	CH_3	H	CH_3
γ-Tocopherol	7,8-Dimethyltocol	γ-T	H	CH_3	CH_3
δ-Tocopherol	8- Methyltocol	δ-T	H	H	CH_3

a = 2 - methyl-2-(4′,8′,12′-trimethyltridecyl) chroman-6-ol



Compound II

Tocopherols

Trivial Name	Chemical Name	Abbreviation	Ring Position R[1]	R[2]	R[3]
Tocotrienol	b	–	H	H	H
α-Tocotrienol	5,7,8-Trimethyltocotrienol	α-T3	CH_3	CH_3	CH_3
β-Tocotrienol	5,8-Dimethyltocotrienol	β-T3	CH_3	H	CH_3
γ-Tocotrienol	7,8-Dimethyltocotrienol	γ-T3	H	CH_3	CH_3
δ-Tocotrienol	8-Methyltocoltrienol	δ-T3	H	H	CH_3

b = 2-methyl-2-(4′,8′,12′-trimethyltrideca-3′,7′,11′-trienyl) chroman-6-ol

Figure 3.1 Structural interrelationships of the tocopherols and tocotrienols.

by the number and location of methyl groups on the chromanol ring. α-Homologs contain three methyl groups, and β- and γ-homologs are dimethylated positional isomers. α-T and δ-T3 are monomethylated.

The tocopherols possess three asymmetric carbons (chiral centers) at position 2 of the chromanol ring and at positions 4′ and 8′ of the phytyl side-chain. Synthetic α-T (all-*rac*-α-T) is a racemic mixture of equal parts of each stereoisomer. Therefore, each tocopherol has eight (2^3) possible optical isomers. Only *RRR*-tocopherols are found in nature. The eight isomers of all-*rac*-α-T (*RRR*-, *RSR*-, *RRS*-, *RSS*-, *SRR*-, *SSR*-, *SRS*-, and *SSS*-) are depicted in Figure 3.2.

The tocotrienols arising from 2-methyl-2-(4′,8′,12′-trimethyltrideca-3′,7′,11′-trienyl) chroman-6-ol (nonmethylated ring structure) have only one chiral center at position 2. Consequently, only 2*R* and 2*S* stereoisomers are possible. Unsaturation at positions 3′ and 7′ of the phytyl side chain permits four *cis*/*trans* geometric isomers. The eight potential tocotrienol isomers are given in Table 3.3. Only the 2*R*, 3′-*trans*, 7′-*trans* isomer exists in nature. Isolation and elucidation of the structural properties of the tocotrienols were accomplished in the 1960s by the research groups of Pennock and associates and Isler and associates.[47,58] Drotleff and Ternes[59] examined hydrogenation of oils and biohydrogenation in the rumen as possible sources for *cis*/*trans* isomerization of the tocotrienols but found little evidence of changes in the 2*R*, *trans*–*trans* configuration.

Vitamin E is fat-soluble and soluble in organic fat solvents. All forms are colorless to pale yellow viscous oils.

In this chapter, the abbreviations α-T and α-T3 will be used for α-tocopherol and α-tocotrienol, respectively. Likewise, β-, γ-, δ-homologs and their corresponding tocotrienols will be abbreviated in a similar manner.

3.2.1.2 Nomenclature rules

Because of the complexity of tocopherol and tocotrienol nomenclature, the IUPAC-IUB 1981 recommendations are given as presented by the Joint Commission on Biochemical Nomenclature.[55–57]

1. Terms
   Vitamin E: The term *vitamin E* should be used as the generic descriptor for all tocol and tocotrienol derivatives exhibiting qualitatively the biological activity of α-tocopherol. This term should be used in derived terms such as *vitamin E deficiency*, *vitamin E activity*, and *vitamin E antagonist*.
   Tocol: The term *tocol* is the trivial designation for 2-methyl-2-(4′,8′,12′-trimethyltridecyl) chroman-6-ol [Compound I (Figure 3.1)], where $R^1 = R^2 = R^3 = H$.
   Tocopherol(s): The term *tocopherol*(*s*) should be used as a generic descriptor for all mono, di, and trimethyl tocols. Thus, the term is not synonymous with the term *vitamin E*.
2. Compound I (Figure 3.1) ($R^1 = R^2 = R^3 = CH_3$), known as α-tocopherol, is designated α-tocopherol or 5,7,8-trimethyl tocol.
3. Compound I (Figure 3.1) ($R^1 = R^3 = CH_3$; $R^2 = H$), known as β-tocopherol, is designated β-tocopherol or 5,8-dimethyl tocol.
4. Compound I (Figure 3.1) ($R^1 = H$; $R^2 = R^3 = CH_3$), known as γ-tocopherol, is designated γ-tocopherol or 7,8-dimethyl tocol.
5. Compound I (Figure 3.1) ($R^1 = R^2 = H$; $R^3 = CH_3$) is known as δ-tocopherol or 8-methyl tocol.
6. Compound II (Figure 3.1) ($R^1 = R^2 = R^3 = H$) 2-methyl-2-(4′,8′,12′-trimethyltrideca-3′,7′,11′-trienyl) chroman-6-ol is designated tocotrienol [only all-*trans* (E,E)-tocotrienols have been found in nature].

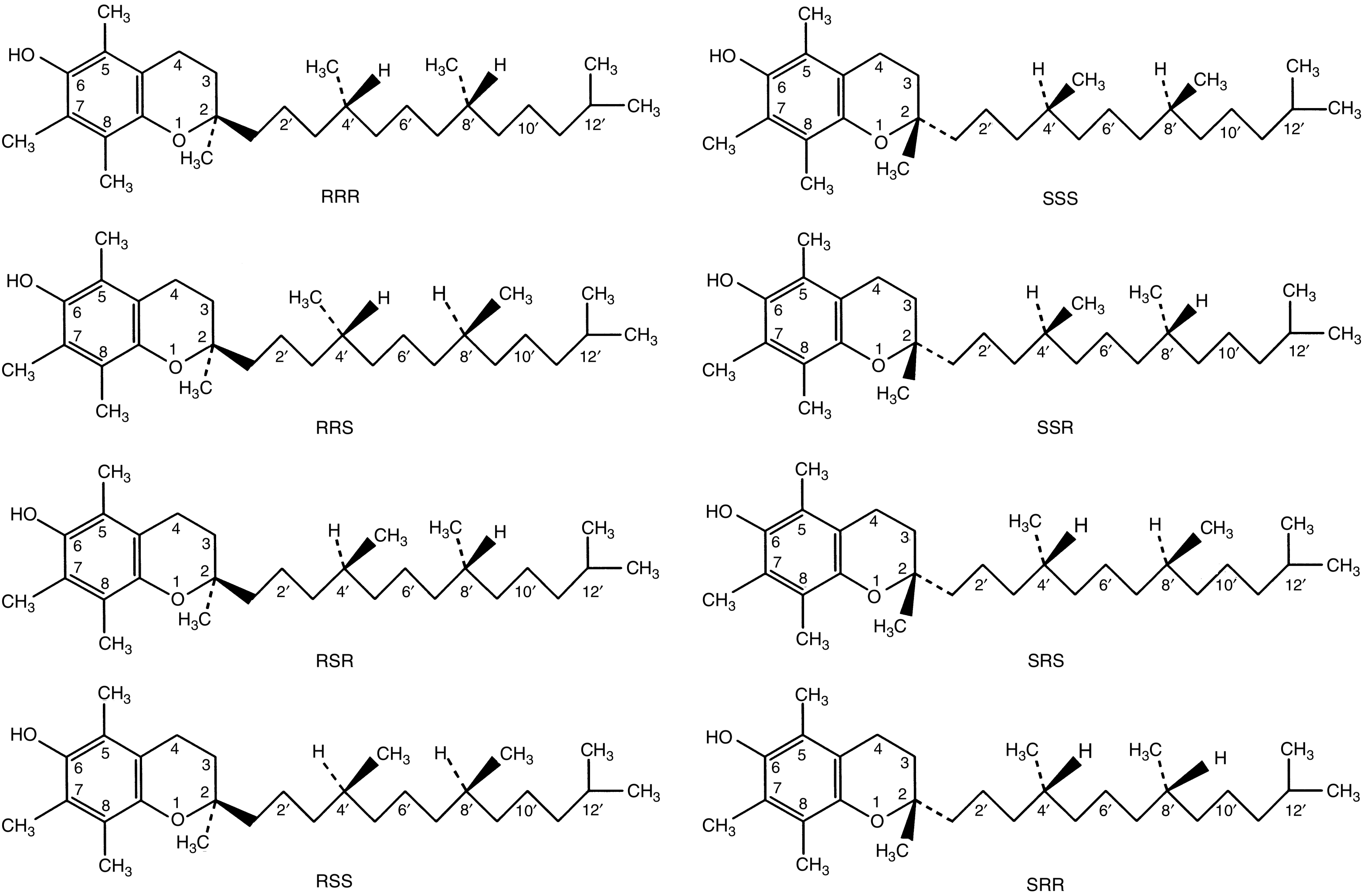


Figure 3.2 Stereoisomers of α-tocopherol.

Table 3.3 The Eight Possible *RS*, *cis/trans* Isomers of the Tocotrienols

R configuration position 2	*S* configuration position 2
2R, 3′*cis*, 7′*cis*	2S, 3′*cis*, 7′*cis*
2R, 3′*cis*, 7′*trans*	2S, 3′*cis*, 7′*trans*
2R, 3′*trans*, 7′*cis*	2S, 3′*trans*, 7′*cis*
2R, 3′*trans*, 7′*trans*	2S, 3′*trans*, 7′*trans*

7. Compound II (Figure 3.1) ($R^1 = R^2 = R^3 = CH_3$), formerly known as ζ, or ζ_2-tocopherol, is designated 5,7,8-trimethyltocotrienol or α-tocotrienol. The name *tocochromanol-3* has also been used.
8. Compound II (Figure 3.1) ($R^1 = R^3 = CH_3$; $R^2 = H$), formerly known as ε-tocopherol, is designated 5,8-dimethyltocotrienol or β-tocotrienol.
9. Compound II (Figure 3.1) ($R^1 = H$; $R^2 = R^3 = CH_3$), formerly known as η-tocopherol, is designated 7,8-dimethyltocotrienol or γ-tocotrienol. The name *plastochromanol-3* has also been used.
10. Compound II (Figure 3.1) ($R^1 = R^2 = H$; $R^3 = CH_3$) is designated 8-methyltocotrienol or δ-tocotrienol.
11. The only naturally occurring stereoisomer of α-tocopherol hitherto discovered has the configuration 2*R*,4′*R*,8′*R* according to the sequence rule. Its semisystematic name is, therefore (2*R*,4′*R*,8′*R*)-α-tocopherol. The same system can be applied to all other individual stereoisomers of tocopherols.
12. Trivial designations are sometimes desirable to indicate briefly the configuration of important stereoisomers of α-tocopherol and especially, mixtures of such stereoisomers. Some of these materials are of considerable commercial and therapeutic importance. The use of the following trivial designations for the most important material of this class is recommended.

    The α-T mentioned earlier, which has the configuration 2*R*,4′*R*,8′*R*, formerly known as d-α-tocopherol, should be called *RRR*-α-T.

    The diastereoisomer of *RRR*-α-T, formerly known as l-α-tocopherol, being the epimer of *RRR*-α-T at C-2 with the configuration 2*R*,4′*R*,8′*R*, should be called 2-*epi*-α-tocopherol.

    A mixture of *RRR*-α-tocopherol and 2-*epi*-α-tocopherol (obtained by synthesis using phytol and the appropriate achiral hydroquinone derivative) should be called 2-*ambo*-α-tocopherol. This mixture was formerly known as *dl*-α-tocopherol until the optical activity of phytol was recognized when *dl*-α-tocopherol was restricted to all-*rac*-α-tocopherol. It is probable that the asymmetric reaction involved in this partial synthesis would only by chance lead to the formation of equimolar proportions. The acetate of 2-*ambo*-α-tocopherol (2-*ambo*-α-tocopheryl acetate) was the former international standard for vitamin E activity.

    The reduction product of natural 5,7,8-tocotrienol, in which the double bonds at 3′, 7′, and 11′ are hydrogenated and the two new asymmetric centers are created at C-4′ and C-8′, is a mixture in unspecified proportions of four diastereoisometric α-tocopherols, having the configurations 2*R*,4′*R*,8′*R*; 2*R*,4′*S*,8′*R*; 2*R*,4′*S*,8′*S*; and 2*R*,4′*R*,8′*S*. The material should be called 4′-*ambo*, 8′-*ambo*-α-tocopherol.

    The totally synthetic vitamin E, obtained without any control of stereochemistry, is a mixture in unspecified proportions (in preparations examined, the proportions closely approached equimolar of four racemates or pairs of enantiomers (i.e., eight diastereoisomers). It should be called all-*rac*-α-tocopherol (it was formerly known

as *dl*-α-tocopherol, although this designation was previously used for 2-*ambo*-α-tocopherol).

13. Esters of tocopherols and tocotrienols should be called *tocopheryl esters* and *tocotrienol esters*, respectively (e.g., α-tocopheryl acetate, α-tocotrienyl acetate).

Biologically, vitamin E functions as the primary antioxidant and as a peroxy free radical scavenger. It is the primary, lipid-soluble, chain-breaking antioxidant that combines actions with other lipid- and water-soluble antioxidants to provide cells with an efficient defense against free radical damage. Free radicals are chemical species capable of independent existence that contain one or more unpaired electrons.[60] Free radical generation occurs when organic molecules undergo homolytic cleavage of covalent bonds and each fragment retains one electron of the original bonding electron pair. Two free radicals are produced from the parent molecule with net negative charges. The free radicals have the ability to react with an electron of opposite spin from another molecule. Free radical generation also occurs when a nonradical molecule captures an electron from an electron donor. α-Tocopherol is located in the cell membranes and protects lipoproteins. It scavenges peroxy free radicals, protecting unsaturated fatty acids. Lipid-generated free radicals have greater affinity for reaction with α-T than with unsaturated fatty acids located within the cell membrane. It is an efficient chain-breaking antioxidant since it can rapidly transfer the phenolic H^+ at C-6 to lipid peroxy radicals. The α-T becomes the α-tocopheroxyl radical, which is stabilized by resonance.

Potency of α-T as an antioxidant depends upon its molecular properties and orientation within the cell membrane. In the membrane, the phytyl side chain is embedded within the bilayer with the chromanol ring and the 6-hydroxyl positioned toward the surface of the membrane. Hydrogen bonding and hydrophobic interactions between the chromanol ring, the phytyl tail, and fatty acids stabilize the membrane and position the chromanol ring to facilitate hydrogen atom donation to lipid peroxy radicals. The α-tocopheroxyl radical migrates from the lipid bilayer to the surface of the membrane, facilitating regeneration of α-T by ascorbic acid and other water-soluble reducing agents that act as hydrogen donors to the α-tocopheroxyl radical.

Nonautoxidative roles for α-T have been recently delineated, which cannot be fulfilled by other tocopherols or tocotrienols. These functions at the molecular level appear to be highly significant to understanding the onset of many chronic diseases. Several critical reviews exist that indicate that the molecular control aspects of vitamin E are just beginning to be understood.[61–65] α-Tocopherol acts as a cell signaling molecule at the posttranscriptional level or at the gene expression level. Many of these cell-signaling functions of α-T are operative through inhibition of protein kinase C (PKC). PKC enzymes are phospholipid-dependent serine/threonine kinases that participate in regulation of cell growth, death, and stress responsiveness.[66] α-Tocopherol acts at the posttranscriptional level by activating protein phosphatase PP_2A, which dephosphorylates PKC.[67,68] Some specific physiological responses regulated by PKC include cell proliferation, platelet adhesion and aggregation, immune response, free radical production, and gene expression. Now, regulation of gene expression at the transcriptional stage is accepted as a primary regulatory function of α-T.

Interest in the tocotrienols from a health-promoting standpoint has greatly increased in recent years. An inhibitor of cholesterol biosynthesis was isolated from barley and proven to be α-T3.[69] Qureshi et al.[69] and Tan et al.[70] conducted human studies with Palmvitee, a T3-rich fraction from palm oil. Both studies led to a reduction of LDL-cholesterol. γ-T3, δ-T3, and α-T3 are effective inhibitors of cholesterol synthesis in rat hepatocytes; whereas, α-T exhibits no inhibition.[71] Other research reported anticarcinogenic effects for the tocotrienols.[72–74] Isoprenoid constituents of the diet have been suggested to suppress tumor growth by depriving the cells of mevalonate-derived products.[75] The hypercholesterolemic

and anticarcinogenic properties of the tocotrienols led Hendrich et al.[76] to suggest that the tocotrienols should be considered as a nutrient independent of the tocopherols. Data reported by Ong[77] indicated that palm oil is the only edible oil consumed in quantity that provides tocotrienols. Cereal oils (wheat, barley, oats, and rice bran) contain tocotrienols but their low consumption limits their significance as vitamin E sources. Cereals and legumes represent other sources of tocotrienols in the Western diet.

Eitenmiller and Lee[43] presented a review of nonantioxidant functions of the tocotrienols and several excellent reviews exist that detail potential roles of the tocopherols and tocotrienols in prevention of many chronic diseases.[78–83]

3.2.1.3 Spectral properties

Ultraviolet (UV) and fluorescence properties of several vitamin E compounds are given in Table 3.4.[84–92] The UV spectra for tocopherols and tocotrienols in ethanol show maximal absorption between 292 and 298 nm. Minimal absorption occurs between 250 and 260 nm.[89–92] Esterification at the 6-hydroxyl shifts the absorption to shorter wavelengths, and all-*rac*-α-tocopheryl acetate shows maximal absorption at 286 nm.[91,92] Intensity of absorption decreases with esterification. $E^{1\%}_{1cm}$ values in ethanol for all-*rac*-α-tocopheryl acetate range from 40 to 44, as compared to 75.8–91.4 for the tocopherols and tocotrienols.[88]

Excitation of the chroman ring at wavelengths near or at maximal absorption (e.g., 292 nm) produces maximal emission at 320 nm or slightly higher wavelengths.[93] The tocopherols and tocotrienols, therefore, possess strong native fluorescence that provides an ideal specific mode of detection for fluorescence-based LC methods. Vitamin E esters show only weak fluorescence compared to the alcohols; however, the fluorescence is strong enough to allow quantitation by LC methods.

Characterization of other physicochemical properties of vitamin E including infrared, nuclear magnetic resonance, and mass spectra can be obtained from a variety of literature sources. Characterization studies were reviewed in several of the previously cited publications.[89–92] Characteristic UV absorption spectra of the tocopherols and tocotrienols are shown in Figure 3.3.[94]

3.2.2 Stability

Since vitamin E is a natural antioxidant serving as a chain-breaking peroxyl free-radical scavenger in biological systems, it interacts as an antioxidant in any fat system containing unsaturated fatty acids undergoing oxidation. Oxidative losses can become substantial quite rapidly, and losses are accelerated by light, heat, alkali pH, lipoxidase reactions, and various metals, primarily iron and copper, and by the presence of free radicals in the fat that can initiate autoxidation. In the absence of oxygen, tocopherols and tocotrienols are stable to heat and alkali conditions such as those used to saponify lipid-containing samples.[43]

Because of the interaction of tocopherols and tocotrienols with oxidative events in foods through their function as antioxidants, physical handling associated with harvesting and storage of raw commodities and, then, further processing and marketing can produce significant changes in vitamin E levels. Depending on environmental factors and the oxidative stress placed on the commodity by the required chain of events necessary to deliver the fresh or processed food to the consumer, these changes can be quite severe with the potential for complete loss. Such events leading to loss of vitamin E can be initiated at any point during the harvesting, storage, processing, and marketing chain. Likewise, storage of the product and food preparation by the consumer can have dramatic effects on the retention of vitamin E in the food at the point of consumption. Because of vitamin E's relative instability, a large degree of variability exists in reported vitamin E contents for similar products. Many researchers have documented the stability of tocopherols and tocotrienols under various

Table 3.4 UV and Fluorescence Properties of Vitamin E

Substance[a]	Molar mass	Formula	Spectral characteristics				
			Absorbance[b]			Fluorescence[c]	
			λ max (nm)	$E^{1\%}_{1cm}$	ε	Ex (nm)	Em (nm)
α-T CAS No. 59-02-9 **10159**	430.71	$C_{29}H_{50}O_2$	292	75.8	[3265]	295	320
β-T CAS No. 148-03-8 **9632**	416.69	$C_{28}H_{48}O_2$	296	89.4	[3725]	297	322
γ-T CAS No. 7616-22-0 **9633**	416.69	$C_{28}H_{48}O_2$	298	91.4	[3809]	297	322
δ-T CAS No. 119-13-1 **9634**	402.66	$C_{27}H_{46}O_2$	298	87.3	[3515]	297	322
α-T3 CAS No. 2265-13-4 **9636**	424.67	$C_{29}H_{44}O_2$	292	86.0	[3652]	290	323
β-T3 CAS No. 49-23-3 **9635**	410.64	$C_{28}H_{42}O_2$	292	86.2	[3540]	290	323
γ-T3 CAS No. 14101-61-2	410.64	$C_{28}H_{42}O_2$	297	91.0	[3737]	290	324
δ-T3 CAS No. 25612-59-3	396.61	$C_{27}H_{40}O_2$	297	85.8	[3403]	292	324
α-tocopheryl acetate CAS No. 52225-20-4 (*dl*) CAS No. 58-95-7 (*l*) **10160**	472.75	$C_{31}H_{52}O_3$	286	40–44	[1891–2080]	285	310
α-tocopheryl succinate CAS No. 4345-03-3 **10159**	53079	$C_{33}H_{54}O_5$	286	38.5	[2044]	—	—

[a] Common or generic name; CAS No.: Chemical Abstract Service number; bold print designates the Merck Index monograph number.[86]

[b] Values in brackets are calculated from corresponding $E^{1\%}_{1cm}$ values, in ethanol.

[c] In hexane.

agronomic, storage, processing, and food preparation conditions for many commodities and their processed foods.

Use of edible oils for frying can lead to rapid decreases in vitamin E levels. Incorporation of air during the frying operation and the fact that edible oils are usually polyunsaturated produces an ideal environment for antioxidant action by the vitamin E components of the oil and their subsequent inactivation. Refining of edible oil results in some loss of vitamin E activity. However, plant oils after refining are more stable to oxidation since the refining process effectively removes pro-oxidants. Most vitamin E loss during refining occurs at the deodorization stage of the process.[43] Owing to its instability to oxidation, all analytical procedures must be completed under conditions that ensure the absence of oxygen and

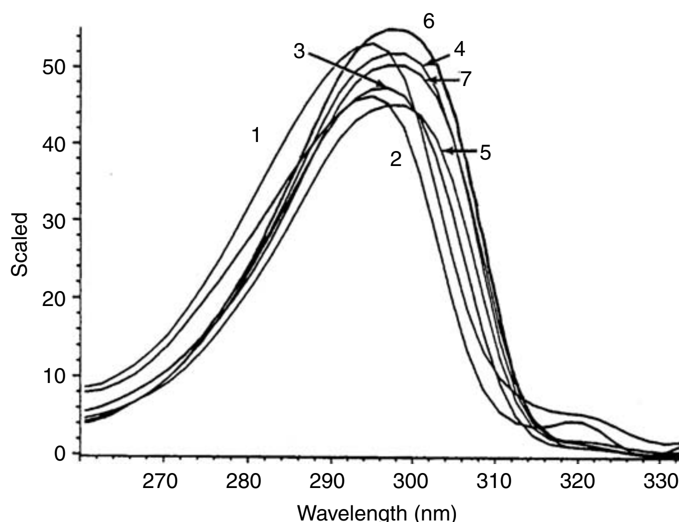


Figure 3.3 UV absorption spectrum of tocopherols and tocotrienols (1 = α-T, 2 = α-T3, 3 = β-T, 4 – γ-T, 5 = γ-T3, 6 = δ-T, 7 = δ-T3). (Reproduced with permission from Tan, B. and Brzuskiewicz, L., *Anal. Biochem.*, 180, 368, 1989. With permission.)

pro-oxidants. The addition of synthetic antioxidants to the sample and extracts at all stages of the analysis is essential (Section 3.3.2.1.1). α-Tocopheryl acetate and α-tocopheryl succinate are stable to oxidation since the hydroxyl group at C-6 is essential to the antioxidant activity of vitamin E. Eitenmiller and Lee provided comprehensive reviews of the stability of vitamin E during food storage, preparation, and food processing operations.[43] These authors cited over 150 journal articles covering many food processing operations and their effects on stability of vitamin E.

3.2.3 *Biological activity*

Initial estimations of the biological activity of the tocopherols and some of the tocotrienols were established by the rat fetal resorption assay, which follows the ability of vitamin E-deficient rats to maintain pregnancy.[95] If vitamin E is not provided during the first 10–15 days after conception, the embryos die and are resorbed. Feeding of known levels of various vitamin E compounds and observation of their effects on fetal survival established relative biological activities. It has been assumed that values of biological activity determined with test animals directly apply to humans. However, with recognition of the selectivity for 2-*R* isomers of α-T through action of the α-TTP, human requirements are now established by using only the 2-*R* isomeric forms of α-T.[23] Since the DRIs refer only to the 2-*R* isomers, confusion exists about currently used units to report vitamin E activity.

An international unit (IU) of vitamin E was defined by the United States Pharmacopeia (USP) as 1 mg of all-*rac*-α-tocopheryl acetate on the basis of biological activity measured by the rat fetal resorption assay.[96] Biological activities of tocopherols, tocotrienols, and synthetic forms of vitamin E are indicated in Table 3.5. After 1980, the USP discontinued the use of the IU and replaced it with USP units derived from the same biological activity values as the IU.[23] Therefore, 1 USP unit is defined as the activity of 1 mg all-*rac*-α-tocopheryl acetate, which equals the activity of 0.67 mg of *RRR*-α-T or 0.74 mg of *RRR*-α-T acetate. Biological activities relative to *RRR*-α-T (100%) have been a convenient way to compare the different forms of vitamin E on the basis of IU or USP units and were used to calculate milligram

Table 3.5 Biological Activity of Natural and Synthetic Vitamin E Forms

Vitamin E forms	Biological activity[a]	
	USP units (IU) mg^{-1}	Compared to *RRR*-α-T (%)
Natural vitamin E (*RRR*-)		
α-Tocopherol	1.49	100
β-Tocopherol	0.75	50
γ-Tocopherol	0.15	10
δ-Tocopherol	0.05	3
α-Tocotrienol	0.75	50
β-Tocotrienol	0.08	5
γ-Tocotrienol	Not known	Not known
δ-Tocotrienol	Not known	Not known
Synthetic		
2*R*,4′*R*,8′*R* α-Tocopherol	1.49	100
2*S*,4′*R*,8′*R* α-Tocopherol	0.46	31
all-*rac*-α-Tocopherol	1.10	74
2*R*,4′*R*,8′*S* α-Tocopherol	1.34	90
2*S*,4′*R*,8′*S* α-Tocopherol	0.55	37
2*R*,4′*S*,8′*S* α-Tocopherol	1.09	73
2*S*,4′*S*,8′*R* α-Tocopherol	0.31	21
2*R*,4′*S*,8′*R* α-Tocopherol	0.85	57
2*S*,4′*S*,8′*S* α-Tocopherol	1.10	60
RRR-α-Tocopheryl acetate	1.36	91
RRR-α-Tocopheryl acid succinate	1.21	81
all-*rac*-α-Tocopheryl acetate	1.00	67
all-*rac*-α-Tocopheryl acid succinate	0.89	60

[a] USP: United States Pharmacopeia; IU: international unit; α-T: α-tocopherol.

Source: Pryor, W. A., Vitamin E Abstracts, VERIS (The Vitamin E Research and Information Service), LaGrange, IL, 1995, p. VII.

α-tocophol equivalent (mg α-TE) values for reporting vitamin E values. International units are still used in food fortification and labeling of supplements; however, their use should be discontinued. Most applications in the Code of Federal Regulations (CFR) rely on the IU to specify regulatory statements pertaining to vitamin E. For example, IUs are used to specify the vitamin E content of infant formula.[98] The USP units are commonly used by the pharmaceutical industry to label vitamin supplements.[23]

The Institute of Medicine, *Panel on Dietary Antioxidants and Related Compounds*,[23] recommended that USP units be redefined by USP to take into account the fact that all-*rac*-α-T has only 50% of the actiivity *RRR*-α-T present in nature or with other *2R*-isomers found in all-*rac*-α-T preparations that are used for food fortification and in supplements.[23] The selectivity for *RRR*-α-T and other *2R*-isomers of α-T provided by α-TTP and studies showing that *2S*-isomers are maintained by the human strongly support this approach to establishment of human requirements. Factors to convert USP units (IUs) to mg *RRR*-α-T or other *2R*-isomers of α-T are given in Table 3.6. Derivation of the conversion factors given in Table 3.6 follows the general formula:

$$\text{Molar conversion factor } (\mu\text{mol IU}^{-1}) = \frac{\text{USP conversion factor (mg IU}^{-1}) \times 1000\ (\mu\text{mol mol}^{-1})}{\text{molecular weight (mg mol}^{-1})}$$

Table 3.6 Conversion Factors to Calculate α-Tocopherol from International Units or United States Pharmacopeia Units to Meet Dietary Reference Intakes for Vitamin E

	USP unit (IU)[a] mg^{-1}	mg USP unit^{-1} (IU^{-1})	μmol USP unit^{-1} (IU^{-1})	α-Tocopherol mg USP unit^{-1} (IU^{-1})
Natural vitamin E				
RRR-α-Tocopherol	1.49	0.67	1.56	0.67
RRR-α-Tocopheryl acetate	1.36	0.74	1.56	0.67
RRR-α-Tocopheryl acid succinate	1.21	0.83	1.56	0.67
Synthetic vitamin E				
all-*rac*-α-Tocopherol	1.10	0.91	2.12	0.45
all-*rac*-α-Tocopheryl acetate	1.00	1.00	2.12	0.45
all-*rac*-α-Tocopheryl acid succinate	0.89	1.12	2.12	0.45

[a] USP: United States Pharmacopeia; IU: international unit.

Source: Institute of Medicine, Food and Nutrition Board, Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids, National Academy of Sciences Press, Washington, D.C., 2000, Chap. 6.

The formula for calculation for *RRR*-α-tocopheryl acetate is

$$\text{Molar conversion factor } (\mu\text{mol IU}^{-1}) = \frac{\text{USP conversion factor (mg IU}^{-1}) \times 1000\ (\mu\text{mol mol}^{-1})}{\text{molecular weight (mg mol}^{-1})}$$

$$= \frac{0.735\ (\text{mg IU}^{-1}) \times 1000\ (\mu\text{mol mol}^{-1})}{472\ (\text{mg mol}^{-1})}$$

$$= 1.56\ (\mu\text{mol IU}^{-1})$$

$$\alpha\text{-T conversion factor (mg IU}^{-1}) = \frac{\text{molar conversion factor } (\mu\text{mol IU}^{-1}) \times 430\ (\text{mg mol}^{-1})}{1000\ (\mu\text{mol mol}^{-1}) \times R}$$

where $R = 2$ for synthetic vitamin E and esters, $R = 1$ for natural vitamin E and esters. So, the α-T conversion factor for *RRR*-α-tocopheryl acetate is determined as follows:

$$\alpha\text{-T conversion factor (mg IU}^{-1}) = \frac{\text{molar conversion factor } (\mu\text{mol IU}^{-1}) \times 430\ (\text{mg mol}^{-1})}{1000\ (\mu\text{mol mol}^{-1}) \times R}$$

$$= \frac{1.56\ (\mu\text{mol IU}^{-1}) \times 430\ (\text{mg mol}^{-1})}{1000\ (\mu\text{mol mol}^{-1}) \times 1}$$

$$= 0.67\ (\text{mg IU}^{-1})$$

3.3 Methods

3.3.1 General approach

Many useful reviews are available on assay methods for tocopherols and tocotrienols in foods and other biologicals.[43,99–112] Older procedures such as colorimetric, spectrophotometric, spectrofluorometric, polarimetric, and thin-layer and open-column chromatographic methods are adequately covered from a historical and applications standpoint. A well-referenced historical background of the development of analytical methods for vitamin E analysis was presented by Eitenmiller and Lee.[43] Parrish,[146] in an early review of methods, classified vitamin E analysis into the following categories:

Biological assays: Fertility tests including resorption-gestation, development of encephalomalacia in newly hatched chicks, development of muscular dystrophy

and creatinuria in rabbits, vitamin E content of blood or liver in various species, and hemolysis of red blood cells in vitamin E-deficient rats.

Physicochemical methods: Ultraviolet, fluorometric, and colorimetric methods.

Chromatographic methods: Paper, thin-layer, column, gas chromatography (GC), and liquid chromatography (LC).

This characterization presented in 1980 is still pertinent for analysis of vitamin E although advanced spectroscopic and LC procedures coupling LC resolution with MS detection are now available.

3.3.2 Regulatory and handbook methods

Analytical procedures for the tocopherols and tocotrienols rapidly advanced from their initiation in the 1930s as colorimetric and open-column chromatography methods were mostly replaced in the 1960s by GC methods. During the next decade, collaborated GC-based methods were made available for regulatory purposes requiring validated methods for routine use. These methods, along with colorimetric and other assay methods, are still considered valid methods and maintain their proper place in accepted regulatory handbooks and methods compendiums. Such methods that are available internationally are summarized in Table. 3.7.

3.3.2.1 AOAC International

The Association of Official Analytical Chemists (AOAC) International *Official Methods of Analysis*[114] provides several methods based on older, chemical approaches. These include colorimetric and polarimetric methods. AOAC Official Method 971.30 (45.1.24) "alpha-Tocopherol and alpha-Tocopheryl Acetate in Foods and Feeds" and AOAC Official Method 948.26 (45.1.26), "alpha-Tocopherol Acetate (Supplemental) in Foods and Feeds" require saponification and quantitation of α-T by thin-layer chromatography (971.30) or after open-column chromatography, colorimetrically, with bathophenanthroline (948.26). If the isomeric form of α-tocopheryl acetate is unknown, AOAC Method 975.43 is a polarimetric method that measures the optical rotation of ferricyanide oxidation product of α-T present after saponification of the sample extract. Optical rotation is negligible for all-*rac*-α-T and positive for *RRR*-α-T. It is necessary to know the isomeric form of supplemental α-tocopheryl acetate to correctly calculate biological activity in IUs or mg *RRR*-α-T (Section 3.2.3). Method 975.43 is only applicable to concentrated supplements, which are concentrated to ≥200 mg α-T g^{-1} before the ferricyanide oxidation step. In terms of utility, the above methods are extremely cumbersome and subject to analytical error owing to their complexity. Although not available as AOAC International collaborated methods for the above matrices, LC methods have largely replaced the colorimetric and polarimetric procedures.

AOAC International provides the following GC procedures for concentrates and drugs:

1. AOAC Official Method 988.14 (45.1.27) "Tocopherol Isomers in Mixed Tocopherols Concentrates"
2. AOAC Official Method 989.09 (45.1.28) "α-Tocopheryl Acetate in Supplemental Vitamin E Concentrates"
3. AOAC Official Method 969.40 (45.1.29) "Vitamin E in Drugs"

Desai and Machlin[99] give a thorough procedural guide to method 969.40. They stress that the method is limited to pharmaceutical products without interfering material. The method resolves *RRR*- and all-*rac*-α-tocopherols and the acetate and succinate esters. Either internal or external standard methodology can be used.

Table 3.7 Regulatory and Handbook Methods for Analysis of Vitamin E

Source	Form	Methods and applications	Approach	Most current cross-reference
***U.S. Pharmacopeia, National Formulary*, 2006, USP 29/NF 24 Dietary Supplements Official Monograph**[96]				
1. Pages 2258–2260	All-*rac*- or *RRR*-α-tocopherol All-*rac*- or *RRR*-α-tocopheryl acetate All-*rac*- or *RRR*-α-tocopheryl succinate	Vitamin E Vitamin E in preparations and capsules	GC Flame ionization	None
2. Pages 2388, 2389–2390	All-*rac*- or *RRR*-α-tocopherol All-*rac*- or *RRR*-α-tocopheryl acetate All-*rac*- or *RRR*-α-tocopheryl succinate	Vitamin E in oil-soluble vitamin capsules/tablets	LC 254 nm	None
3. Pages 2391, 2394–2395, 2397, 2402, 2410	All-*rac*- or *RRR*-α-tocopherol All-*rac*- or *RRR*-α-tocopheryl acetate All-*rac*- or *RRR*-α-tocopheryl succinate	Vitamin E in oil- and water-soluble vitamin capsules/tablets, oral solution w/wo minerals	LC	None
4. Page 3448–3449	*RRR*-α-tocopherol *RRR*-β-tocopherol *RRR*-γ-tocopherol *RRR*-δ-tocopherol	Tocopherols Excipient	GC Flame ionization	None
British Pharmacopoeia, 2007[113]				
1. Pages 2059–2061	All-*rac*-α-tocopherol	α-Tocopherol	GC Flame ionization	None
2. Pages 2061–2062	*RRR*-α-tocopherol	*RRR*-α-tocopherol	GC Flame ionization	None
3. Pages 2062–2063	All-*rac*-α-tocopheryl acetate	α-Tocopheryl acetate	GC Flame ionization	None
4. Pages 2063–2064	*RRR*-α-tocopheryl acetate	*RRR*-α-tocopheryl acetate	GC Flame ionization	None
5. Pages 2065–2066	α-Tocopheryl acetate concentrate (powder form)	α-Tocopheryl acetate	GC Flame ionization	None
6. Pages 2066–2068	α-Tocopheryl hydrogen succinate	All-*rac*-α-tocopheryl succinate	GC Flame ionization	None
7. Pages 2068-2070	*RRR*-α-tocopheryl hydrogen acetate	*RRR*-α-tocopheryl acetate	GC Flame ionization	None

Continued

Table 3.7 (Continued)

Source	Form	Methods and applications	Approach	Most current cross-reference
***AOAC Official Methods of Analysis*, 18th ed., 2005**[114]				
1. 45.1.24	All-*rac*- or *RRR*-α-tocopherol All-*rac*- or *RRR*-α-tocopheryl acetate	AOAC Official Method 971.30 α-Tocopherol and α-Tocopheryl Acetate in Foods and Feeds Colorimetric Method	Colorimetric 534 nm	*J. Assoc. Off. Anal. Chem.*, 54, 1, 1971[115]
2. 45.1.25	All-*rac*- or *RRR*-α-tocopherol All-*rac*- or *RRR*-α-tocopheryl acetate	AOAC Official Method 975.43 Identification of *RRR*- or all-*rac*-α-Tocopherol in Drugs and Food or Feed Supplements (≥200 mg g^{-1})	Polarimetric	*J. Assoc. Off. Anal*. Chem., 58, 585, 1975[116]
3. 45.1.26	All-*rac*- or *RRR*-α-tocopherol All-*rac*- or *RRR*-α-tocopheryl acetate	AOAC Official Method 948.26 α-Tocopheryl acetate (supplemental) in Foods and Feeds	Colorimetric 534 nm	*J. Assoc. Off. Anal. Chem.*, 54, 1, 1971[115]
4. 45.1.27	*RRR*-tocopherol *RRR*-β-tocopherol + *RRR*-γ-tocopherol *RRR*-δ-tocopherol	AOAC Official Method 988.14 Tocopherol isomers in mixed tocopherols concentrate	GC Flame ionization	
5. 45.1.28	All-*rac*-α-tocopheryl acetate	AOAC Official Method 989.09 α-Tocopheryl Acetate in Supplemental Vitamin E Concentrates	GC Flame ionization	*J. Assoc. Off. Anal. Chem.*, 71, 1168, 1988[117]
6. 45.1.29	All-*rac*- or *RRR*-α-tocopherol All-*rac*- or *RRR*-α-tocopheryl acetate All-*rac*- or *RRR*-α-tocopheryl succinate	AOAC Official Method 969.40 Vitamin E in Drugs	GC Flame ionization	*J. Assoc. Off. Anal. Chem.*, 61, 475, 1978[118]
7. 50.1.04	All-*rac*-α-tocopheryl acetate as α-tocopherol	AOAC Official Method 992.03 Vitamin E Activity (all-*rac*-α-tocopherol) in Milk-Based Infant Formula	HPLC 280 nm	*J. AOAC Int.*, 76, 399, 1993[119]
***European Committee for Standardization*, 2000**[120]				
1. EN 12822	*RRR*-tocopherols	Foods	LC Fluorescence Ex λ = 295 Em λ = 330	*J. AOAC Int.*, 79, 902, 1996;[121] Z. *Lebensm. Unters Forsch*, 202, 80, 1996[122]

American Oil Chemists' Society[123]				
1. Ce 7-87	*RRR*-tocopherols	Deodorizer distillate	Capillary GC	*J. AOCS*, 65, 881, 1988;[124] *J. AOCS*, 65, 1936, 1988[125]
2. Ce 8-89	*RRR*-tocopherols *RRR*-tocotrienols	Vegetable oils and fats	LC Fluorescence Ex λ = 290 Em λ = 330	*Pure Appl. Chem.*, 60, 877, 1988[126]
***Food Chemicals Codex*, 5th ed., 2004**[127]				
1. Pages 417–418	All-*rac*-α-tocopherol	All-*rac*-α-tocopherol (NLT 96.0%, NMT 102.0%)	GC Flame ionization	None
2. Page 418	*RRR*-α-tocopherol	All-*rac*-α-tocopherol concentrate (concentrates from edible oil deodorizer distillate)	GC Flame ionization	None
3. Pages 419–420	*RRR*-α-tocopherol *RRR*-β-tocopherol *RRR*-γ-tocopherol *RRR*-δ-tocopherol	Tocopherols concentrate, mixed (concentrate from edible oil deodorizer distillate)	GC Flame ionization	None
4. Pages 420–421	α-Tocopheryl acetate (NLT 96.0%, NMT 102.0%)	*RRR*-α-tocopheryl acetate (acetylization of α-tocopherol from edible oil)	GC Flame ionization	None
5. Pages 421–422	All-*rac*-α-tocopheryl acetate	all-*rac*-α-tocopheryl acetate	GC Flame ionization	None
6. Page 422	*RRR*-α-tocopheryl acetate	*RRR*-α-tocopheryl acetate concentrate	GC Flame ionization	None
7. Pages 422–424	*RRR*-α-tocopheryl succinate	all-*rac*-α-tocopheryl acid succinate	GC Flame ionization	None
***Methods for the Determination of Vitamins in Foods*, COST 91**[128]				
1. Page 91	*RRR*-α-tocopherol or all-*rac*-α-tocopheryl acetate	Foods	LC Fluorescence Ex λ = 293 Em λ = 326	None
2. Page 107	*RRR*-tocopherols *RRR*-tocotrienols	Fats and oils	LC Fluorescence Ex λ = 293 Em λ = 326	None

AOAC International[114] provides one LC-based procedure for the assay of vitamin E activity in milk-based infant formula, AOAC Official Method 992.03 (50.1.04) "Vitamin E Activity (all-*rac*-α-tocopherol) in Milk-Based Infant Formula." The method, collaborated in 1993,[119] utilizes saponification and chromatography on silica with hexane:isopropanol (99.92:0.08) with UV detection at 280 nm. Collaboration of AOAC Method 992.03 was recommended by the AOAC Task Force on Methods for Nutrition Labeling to allow its use with other matrices.[129] However, such studies have not yet been completed. Use of fluorescence detection and better instructions to prevent vitamin E decomposition during saponification would improve the method and help analysts prevent vitamin E loss.

3.3.2.2 American Oil Chemists Society

The American Oil Chemists Society (AOCS)[123] provides a capillary GC method for analysis of total tocopherols in deodorizer distallate (Ce 7-87) and an LC method for assay of fats and oils (Ce 8-89). Method Ce 7-87 "Total Tocopherols in Deodorizer Sludge" is based on a method published by Berner[124] and Marks.[125] Capillary GC, with its development, solved packed-column chromatography of the tocopherols and tocotrienols. Of significance, the capillary GC procedure resolved β– and γ-T, which could not be separated with packed columns. Saponification was not required and sample preparation time was greatly reduced. The collaborative study indicated that the four laboratories of the nine that submitted results obtained good agreement of results between analyses completed on different days; however, results between laboratories and between duplicate vials were poor, producing high RSD_R values. The study suffered because samples degraded during cold storage and because one participating laboratory could not meet time frame requirements.[124] The method has never been fully collaborated by AOCS.

Details of Method Ce 7-87 follow:

Apparatus

Gas chromatograph equipped with FID
30-m DB-5 capillary column, 0.25-μm film thickness, 0.25-mm id

Chromatography

Helium carrier gas, 2 cm^3 min^{-1}

Program

140–300°C at 10°C min^{-1}
Hold 6 min
300–320°C at 5°C min^{-1}
Hold 10 min
Split flow rate 150 cm^3 min^{-1}
Injector temperature—240°C
Detector temperature—345°C

Calculation

Internal standard—heptadecanyl stearate
Method Ce 8-89 "Tocopherols and Tocotrienols in Vegetable Oils and Fats by HPLC" is based on parameters provided by the International Union of Pure and Applied Chemistry (IUPAC) for the analysis of fats and oils.[126] Details of the method include the following:

Apparatus

High performance liquid chromatography (HPLC) equipped with a fluorescence detector
UV spectrometer
Rotary evaporator

Reagents

α-, β-, α, and α-T standards
Methanol
Dichloromethane
Hexane
Isopropanol

Chromatography

Column	25 cm × 4.6 mm
Stationary phase	Microparticulate silica, 5 µm
Mobile phase	Isopropanol in hexane (0.5:99.5)
Column temperature	Ambient
Flow rate	1 mL min^{-1} (0.7–1.5 mL min^{-1})
Injection	20 µL
Detection	Fluorescence, Ex λ = 290, Em λ = 330; or UV at 292 nm (not preferred)
Calculation	Peak area, external standard

3.3.2.3 European committee for standardization

The European Committee for Standardization, Technical Committee CEN/TC 275 approved EN12822 "Foodstuffs—Determination of vitamin E by high performance liquid chromatography—Measurement of α–, β–, γ–, and δ-tocopherols" in 2000.[120] The method is based on original work by Konings and colleagues.[121] Extraction of the vitamin E components is by saponification or solvent extraction with normal-phase chromatography on Si60 with fluorescence detection (Ex λ = 295, Em λ = 330). The procedures allow use of UV detection at 292 nm, but fluorescence detection is recommended to avoid chromatogram interferences. The method is presented in EN12822 standard method protocol in a clear manner, providing many essential details to the analyst. Specific extraction methods are provided for oil and fats, butter and margarine, and other foods as a group. RSD_r values were 4.1% for margarine and 4.0% for milk powder. RSD_R values for the same products were 6.1% and 7.0%, respectively.

3.3.3 Advances in analysis of the tocopherols and tocotrienols

3.3.3.1 Gas chromatography

Gas chromatographic methods for assay of vitamin E were developed before the advent of LC. GC methods applicable to determination of vitamin E in biologicals have been thoroughly discussed by Nelis and colleagues[100,102] and Lang and colleagues.[101] Early methods were hampered by the inability of packed-column chromatography to resolve β- and γ-tocopherols and β- and γ-tocotrienols. In addition, packed-column chromatography was labor-intensive and affected by interferences to both the tocopherols and the internal standard peaks, requiring saponification to reduce the interferences and the use of correction factors to correct for unremoved interferences.[125] Lang and colleagues[101] stated that through 1988, no packed-column GC method had been developed to adequately resolve β- and γ-T or β– and γ-tocotrienols (γ-T3). Also, α-T3 and α-T were not well resolved. Development of capillary GC solved many of the problems associated with packed-column chromatography. GC resolution of β- and γ-T, and β- and γ-T3 can only be efficently accomplished as TMS derivatives on capillary columns.

Although LC is now universally accepted as an easy and accurate approach for quantitation of vitamin E homologs, GC methods provide an alternative approach for analysis of complex matrices. Such procedures usually rely on the linking of GC and mass spectrometry (GC-MS).[130–140] As an example, in 1998, Frega et al.[130] identified and confirmed the

presence of several components of annatto, including α- and β-T3. Analysis of the total lipid fraction included saponification, treatment of the extract with diazomethane to methylate free fatty acids, and silanization. Gas chromatography was on the 30-m capillary column containing SPB-5 as the stationary phase.

Mottier et al.[140] published a GC-MS and a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for quantitation of α-T and α-tocopherolquinone in human plasma. For GC-MS assay, the analytes were converted to TMS-derivatives and monitored in the selective ion mode. With the LC-MS/MS procedure, the analytes were detected using multiple reactions monitoring after positive electrospray ionization (ESI). Both methods used isotopically labeled (deuterated) forms of the analytes as internal standards. The GC-MS and LC-MS/MS-ESI procedures showed similar accuracy and within-day precisions. The LC-MS/MS method was faster and more sensitive.

3.3.3.2 High-performance liquid chromatography

High-performance liquid chromatography (HPLC) was first applied to the resolution of vitamin E and other fat-soluble vitamins in the 1971 by Schmit et al.[141] and Williams et al.[142] Two reversed-phase packing materials, Permaphase ODS and Zipax HCP, introduced by DuPont in the early stages of LC, were used to study resolution of the fat-soluble vitamins, including α-T and α-tocopheryl acetate. Permaphase ODS was a C_{18} column and Zipax HCP was a hydrocarbon coating on Zipax support. Good resolution of the tocopherols in a mixed tocopherol concentrate was obtained with the ODS column, although the peaks other than α-T were not identified because the only standard available to the research group was α-T. α-Tocopherol and all-*rac*-α-tocopheryl acetate could be resolved from other fat-soluble vitamins. The mobile phases studied included methanol:water combinations with either isocratic or gradient elution. Ultraviolet detection at 254 nm was sufficient to detect the fat-soluble vitamins for the quite concentrated preparations used in the study.

Van Niekerk[143] published a study in 1973 that showed the power of LC for vitamin analysis and led the way for its rapid advance in the next few years. Van Niekerk's work was the first published LC paper dealing with food analysis of vitamin E. Also, this study used normal-phase chromatography on Corasil II with the mobile phase of isopropanol ether:hexane (5:95). Van Niekerk's work set important principles for the application of LC to vitamin E analysis that eased the work of later vitamin E analysts. These included the following:

1. Oils could be injected directly onto a silica column; therefore, no sample preparation other than dilution of the oil was required.
2. Fluorescence provided an ideal, sensitive, and specific detection mode.
3. Positional isomers, β- and γ-T, could be resolved.
4. Good reproducibility was possible.
5. Recoveries of added tocopherols to oils were high, approaching 100%.
6. The procedure was "fast and easy." Van Niekerk predicted that HPLC would find wide application for the routine assay of vitamin E in foods.

Other early applications of LC to vitamin E analysis of food include research by Cavins and Inglett[144] and Abe et al.[145] Cavins and Inglett[144] applied the method to corn oil and wheat bran, obtaining clean chromatograms with direct injection of the oil diluted in cyclohexane with UV detection at 254 nm. No quantitation was attempted. The study was significant in that it showed that the eight vitamin E homologs could be resolved from plant oils without cleanup or derivatization and that retention time could be a valuable parameter for analyte identification, considering the resolving power of the technique.

Abe et al.[145] also applied LC to the quantitation of tocopherols in vegetable oils. They used normal-phase chromatography on JASCO-PACK WC-03 with a mobile phase of diisopropyl ether in hexane (2:98) and fluorescence detection. They quantitated α-, β-, γ-, and δ-T

in soybean, cottonseed, and wheat germ oil, thus, providing some of the quantitative data on the vitamin E content of food determined by HPLC. In addition, the study showed that the LC data compared closely with GC data and that dried oil injection provided data that was very comparable to data obtained by saponification of the oils before LC resolution. Unlike with GC, β- and γ-T were resolved by the LC system. The research clearly demonstrated the superiority of fluorescence detection to UV detection in terms of sensitivity. After 1975, LC coupled with fluorescence detection became the method of choice for vitamin E assay of foods and other biological samples.

3.3.3.2.1 Extraction procedures for analysis of vitamin E by LC. A large number of diverse extraction procedures have been used for extraction and quantification of vitamin E as well as other fat-soluble vitamins from biological matrices. Except for quantification of vitamin E in oils, which can be directly injected onto a normal-phase LC column after dilution with n-hexane or mobile phase, the vitamin E must be concentrated and in many cases, freed from the sample matrix. Preparation of the vitamin E fraction usually requires saponification of the entire sample matrix or of an isolated lipid fraction or extraction of the total lipid from the sample with suitable solvent. The following is a brief review of currently used extraction procedures.

3.3.3.2.1.1 Saponification. Saponification is a general term referring to alkaline hydrolysis. Usually, KOH is used, although NaOH is specified by some procedures. The hydrolysis is used to free fat-soluble vitamins, except vitamin K, which is labile under the alkaline environment, from the sample matrix. Hydrolysis results in cleavage of the ester linkages of acylglycerols and liberation from protein, lipid, and carbohydrate complexes; phospholipids are hydrolyzed; tocopherol and tocotrienol esters are hydrolyzed; pigments and other substances that may interfere with the chromatography are removed; and the sample matrix is disrupted, facilitating vitamin extraction.[107,146,147] The procedure includes the following general steps:

1. Addition of ethanolic base (KOH) to the sample together with a suitable antioxidant such as pyrogallol or ascorbic acid or combinations of antioxidants
2. Dispersion of the sample to ensure that clumping does not occur to such an extent that the ethanolic KOH cannot penetrate the sample matrix
3. Flushing of the saponification vessel with inert gas (N_2)
4. Refluxing with the aid of an air condenser (Figure 3.4) under gold fluorescent lighting or in the dark
5. Cooling of the digest
6. Addition of 1% NaCl or water
7. Partitioning of the digest with ether, hexane, ethyl acetate in hexane, or other suitable solvent mixtures
8. Collection of the organic solvent phase
9. Washing of the organic solvent to remove fatty acid soaps
10. Concentration of the nonsaponifiable fraction (vitamin E fraction)

Many saponification procedures have been used with success for vitamin E analysis. Often, exact parameters for a specific matrix must be determined through studies on the specific matrix.

Ball[147] provides a guide for the digestion mixture, which includes 5 ml of 60% w/v aqueous KOH and 15 ml of ethanol per 1 g of fat. Because of the instability of vitamin E under alkaline conditions, care must be taken to avoid destruction of the vitamin E homologs during the saponification. Steps required to avoid undue loss during digestion include flushing of the digestion vessel with nitrogen, addition of antioxidant (pyrogallol or ascorbic acid),

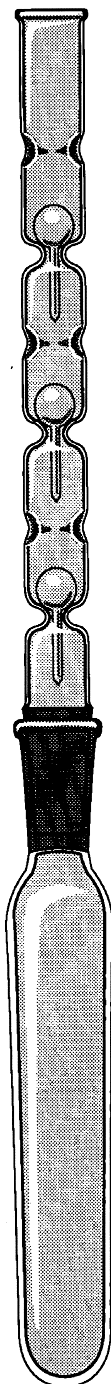


Figure 3.4 Reflux condenser for saponification.

and protection from light. Temperatures and times used for saponification range from ambient temperature for 12 h or longer[148–150] to 70°C for 30 min or less.[151,152] Saponification parameters such as sample size, volumes of alkali and ethanol, and time and temperature can be varied to optimize the digestion. Most efficient saponification and highest recovery

of vitamin E is obtained when the digestion is completed under reflux conditions. Use of an air condenser greatly simplifies the saponification step in vitamin E assay (Figure 3.4).

Following the saponification, the digest is diluted with water or 1% NaCl to inhibit emulsion formation and extracted with ether, petroleum ether, hexane, or ethyl acetate in hexane, or other solvent mixtures. The unsaponifiable components including vitamin E are extracted into the solvent while the fatty acid soaps, glycerols, and many other potentially interfering substances remain in the alkaline aqueous phase.

Problems exist in getting efficient transfer of the tocopherol and tocotrienol homologs into the organic solvent phase from the aqueous phase. Ueda and Igarashi reviewed factors affecting extraction of vitamin E from the saponification medium.[151,153] Significant factors include ethanol concentration of the digest, composition of the extracting solvent, and the level of lipids used in the digest. When *n*-hexane is the extraction solvent, ethanol concentrations must be kept below 30% to ensure complete extraction of δ-T and tocol (an internal standard) and below 15% if 2,2,5,7,8-pentamethyl-6-hydroxy chroman (PMC) is used as the internal standard. Ethanol concentration has no effect on the extraction of α-T and only a slight effect on β- and γ-T.[153]

n-Hexane is the most commonly used solvent for extraction of saponification digests. However, addition of ethyl acetate, which is more polar than *n*-hexane, to levels up to 10% v/v to the *n*-hexane can improve recoveries for β-T, γ-T, and δ-T, tocol, and PMC.[154] Increasing the level of ethyl acetate above 10% causes the volume of the solvent layer to decrease as the solvent becomes more miscible in the aqueous phase; therefore, extracts containing more than 10% ethyl acetate need to be carefully proven for use.

Fatty acid salts from the lipid can increase solubility of the vitamin E homologs in the aqueous phase and decrease extraction into the nonpolar solvent. Ueda and Igarashi,[155] working with corn oil, showed that recovery losses of β-T, γ-T, δ-T, and tocol can be significant. Recovery losses were observed with oil levels as low as 5 mg. Extraction of α-T was not affected by the lipid level in the digest. Recovery of tocol was most severely affected.

For cereals, Panfili et al.[211] showed that saponification provided higher and more reproducible results compared to various direct solvent extraction procedures. With some food matrices, extraction of the lipid before saponification has been advantageous. As an example, Tuan et al.[156] reported that lipid extraction from infant formula before saponification provided extracts with fewer interferences than extracts obtained by saponification of the entire formula. However, in our experience, most samples can be handled with direct saponification without prior fat extraction. Protocols to extract vitamin E from biological samples are often designed to decrease time and solvent requirements and to allow for use of small sample weights when samples are limited.[152,154] Details of published saponification procedures are given in Table 3.8.[149–165]

Procedures of Indyk[152] and Uega and Igarashi[154] are briefly summarized to provide methodology applicable to a wide variety of biological samples.

1. Procedure Applicable to Dairy Products, Food, and Tissues (Indyk[152])

Sample Size: 0.5 g whole milk powder, powdered infant formula, freeze-dried organs, fish, cereal, 5.0 g of fluid milk; 0.1–0.2 g butter, margarine, or vegetable oil.

Procedure: Weigh sample into test tube and add 10.0 mL ethanol containing 1% pyrogallol. Add α-T standard (200 μL of known concentration [20–30 μg 100 mL^{-1} absolute ethanol]) to the unfortified sample to provide a parallel assay for recovery data. Add 2 mL of 50% KOH and loosely stopper the tubes. Incubate at 70°C for 7 min with periodic agitation. Cool the tubes and add 20 mL of light petroleum ether: diisopropyl ether (3:1). Shake mechanically for 5 min. Add 30 mL water, invert ten times, and centrifuge at 180 × g for 10 min. A 10 μL volume of the clear upper layer is injected directly into an isocratic LC system.

Table 3.8 Saponification Conditions Used for Extraction of Vitamin E

Matrix	Hydrolysis sample size	Conditions	Antioxidant	Internal standard or % extractant	%Recovery	References
Feeds	0.5 g	Ethanolic KOH 70°C, 30 min	Pyrogallol	Hexane Diethyl ether Petroleum ether	Tocol—not suitable for addition prior to saponification	*J. Micronutr. Anal.*, 1, 31, 1985[151]
Meat	10 g	Ethanolic KOH Ambient, overnight	Ascorbic acid	Hexane	α-T—97% β-T—100% γ-T—97% δ-T—68%	*J. Agric. Food Chem.*, 33, 1215, 1985[149]
Infant formula	1 g	Ethanolic KOH Reflux, 30 min	Pyrogallol	Hexane	α-T—96–109%	*J. Micronutr. Anal.*, 6, 35, 1989[156]
Dairy products Foods Tissues	10 g	Ethanolic KOH 70°C, 7 min	Pyrogallol	Petroleum ether:isopropyl ether (3:1)	α-T (IS) added to unfortified sample	*Analyst*, 113, 1217, 1988[152]
40 foods	10 g	Ethanolic KOH Ambient, overnight	Ascorbic acid Nitrogen flush	Hexane	>80% for α- and γ-T in all samples	*J. Food Compos. Anal.*, 2, 200, 1989[150]
Infant formula	10 mL	Ethanolic KOH 70°C, 25 min	Pyrogallol	Hexane:methylene chloride (3:1)	None	*J. AOAC Int.*, 76, 399, 1993[119]
Human diets	10–20 g	Ethanolic KOH Ambient, overnight	Ascorbic acid	Hexane	α-T—99% β-T—95% γ-T—99% δ-T—80%	*Int. J. Vit. Nutr. Res.*, 53, 35, 1984[158]

Seeds oils	1–5 g	Ethanolic KOH Reflux, 30 min	Sodium ascorbate	Diisopropyl ether	α-T—93% γ-T—94% α-T3—95%	*J. Food Sci.*, 50, 121, 1985[159]
Tomato Broccoli	7.5 g	Ethanolic KOH Reflux, variable time	Pyrogallol	Hexane	α-T—102%	*J. Food Compos. Anal.*, 13, 45, 2000.[157]
Rye flours	0.5 g	Ethanolic KOH 100°C, 25 min	Ascorbic acid Nitrogen flush	Hexane:ethyl acetate (8:2)	90.3–94.3	*J. Food Compos. Anal.*, 17, 749, 2004[160]
Tropical plants	5 g (dwb)	Ethanolic KOH 80°C, 40 min	Ascorbic acid Nitrogen flush	Hexane:ethyl acetate (9:1)	—	*J. Agric. Food Chem.*, 49, 3101, 2001[161]
Pig muscle	1 g	Ethanolic KOH 80°C, 40 min	Ascorbic acid Nitrogen flush	Isooctane containing 0.01% BHT	α-T—78%	*J. Agric. Food Chem.*, 51, 1120, 2003[162]
Ferula hermonis root (Zalooh)	10 g	Ethanol:methanol: water (4:20:76) with 15 mL 70% NaOH, 60 min, 100°C	Ascorbic acid	Add water, separate organic phase	Standard addition of α-T, regression curve	*J. Food Compos. Anal.*, 18, 607, 2005[163]
Human milk	1 mL	Methanolic KOH 70°C, 30 min	Pyrogallol	Acidify, hexane	δ-T (IS)	*Acta Chromatogr.*, 16, 48, 2006[164]
Moringa oleifera leaves, flowers, beans	0.4 g	Methanolic KOH 80°C, 15 min	Pyrocatechol	Hexane	α-T—90.3% γ-T—89.1%	*J. Chromatogr. A*, 1105, 111, 2006[165]

2. Procedure Applicable to Blood and Tissues. (Ueda and Igarashi154)

Blood: To 200 μL plasma or 400 μL of 50% hematocrit RBC suspension in two 10 mL centrifuge tubes with teflon-coated screwcaps, add 1 mL 6% ethanolic pyrogallol to each tube. Preheat the solution to 70°C for 3 min and to one tube, add 1 mL of an ethanolic solution of PMC (0.3 μg) as an internal standard; to the other tube, add 3 mL of an ethanolic solution containing 3.0 μg each of α-, β-, γ-, and δ-T and PMC. Add 0.2 mL of 60% KOH and saponify at 70°C for 30 min. Cool tubes in ice water and add 4.5 mL of 1% NaCl. The saponification mixture is extracted with 3 mL of 10% ethyl acetate in *n*-hexane. Centrifuge the saponified extracts at 3000 r.p.m. for 5 min and pipet 2 mL of *n*-hexane layer into a 10 mL conical glass tube. Evaporate the *n*-hexane under a stream of nitrogen. Redissolve the unspiked residue in 200 μL of *n*-hexane and the residue from the spiked sample in 2.0 mL of *n*-hexane. For each, inject 10 μL into the LC system.

Tissues: Weigh 100 mg of tissue into a 10 mL centrifuge tube with teflon-coated screwcap. Add 100 μL of 1% NaCl, 1 mL of 6% ethanolic pyrogallol, and 2.0 mL of 60% KOH. Saponify at 70°C for 60 min. Add 4.5 mL of 1% NaCl to the cooled digest and extract with 3 mL of 10% ethyl acetate in *n*-hexane. Centrifuge the saponified extracts at 3000 r.p.m. for 5 min and pipet 2 mL of the *n*-hexane layer into a conical glass tube for concentration under a stream of nitrogen. Redissolve the residue in 200 μL of *n*-hexane and inject 10 μL into the HPLC. Recoveries can be determined by use of a parallel spike or by use of PMC internal standard.

Optimization of saponification conditions: Lee et al.[157] used response surface methodology (RSM) to optimize vitamin E extraction from tomato and broccoli by saponification. Variables examined included the amount of 60% KOH, saponification time, and final ethanol concentration. The optimized parameters were obtained by ridge analysis. On the basis of the ridge analysis, optimal saponification conditions were (a) 8.4–8.9 mL 60% KOH, (b) 50.7–54.3 min at 70°C, and (c) 30.1–35.0% ethanol. All trials used a sample size of 7.5 g. With the optimized parameters, experimental concentrations agreed closely with values predicted by ridge analysis. Effects of KOH amount and final ethanol concentration under constant saponification times on response surface plots of γ-T are shown in Figure 3.5.

The RSM technique allows evaluation of the effects of many factors and their interactions on response variables. Advantages of RSM in optimization studies of all types are the reduced number of experimental trials needed to evaluate multiple parameters and their interactions, labor, and time required to obtain the optimized process. The RSM studies have been widely applied for optimization of processes in the food and pharmaceutical industries. They had not been applied to vitamin extraction optimization to any extent before Lee et al.[157] study, which shows that RSM can be used to optimize vitamin extraction parameters and may be useful to vitamin chemists.

In a later study, Ryynänen et al.[160] applied RSM techniques to optimize extraction of tocopherols and tocotrienols from rye and other cereals. The optimized method used saponification of 0.5 g of rye flour or other cereal flours with 0.5 mL of KOH (50 g 100 mL^{-1}) at 100°C for 25 min in a saponification mixture containing 0.1 g ascorbic acid, 5 mL ethanol, and 2 mL water. After the hot saponification, 2.5 mL of water and 2.5 mL of ethanol were added to the cooled tubes and unsaponified lipids were extracted with 10 mL portions of hexane:ethyl acetate (8:2) (3×). Organic extracts were washed 3× with water with addition of NaCl to avoid emulsion formation, and the washed extract was transferred to a round bottom flask for evaporization.

Recoveries of the procedure ranged from 90.3% to 94.3%. Use of the rapid hot saponification under the optimized conditions provided higher tocopherol and tocotrienol levels compared to ambient, long-time saponification, and direct solvent extraction procedures

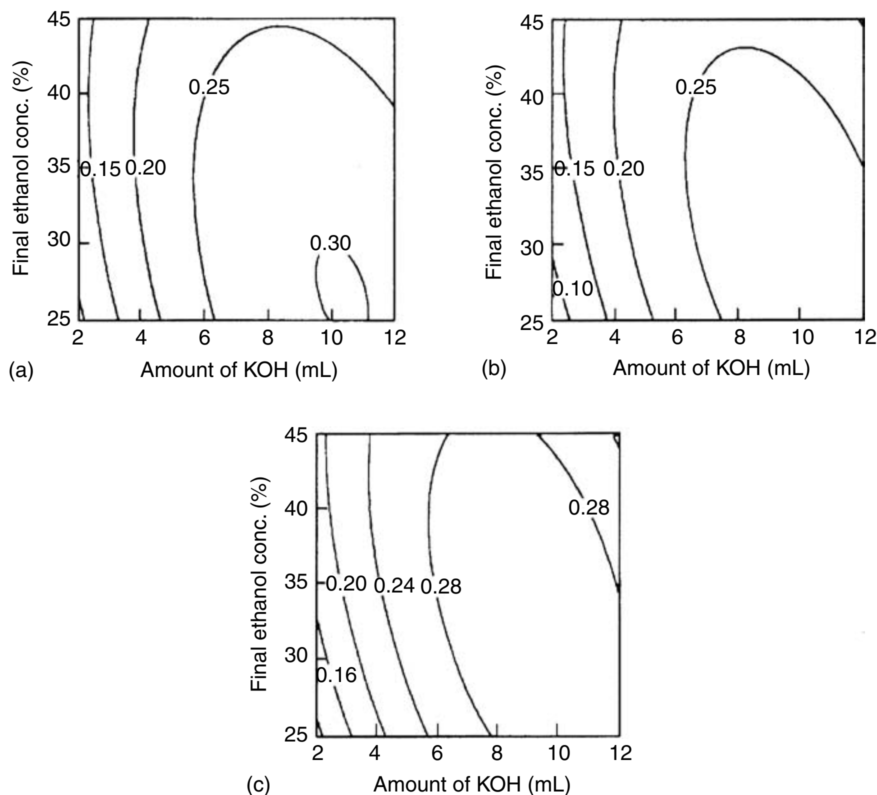


Figure 3.5 Response surface plots of γ-T showing effects of amount of potassium hydroxide and final ethanol concentration under constant saponification time. The numbers inside plots represent mg tocopherol in 100 g sample. Saponification time: (a) 15 min, (b) 35 min, (c) 55 min. (Reproduced from Lee, J., Ye, L., Landen, W. O., Jr., and Eitenmiller R. R., *J. Food Compos. Anal.*, 13, 45, 2000. With permission.)

with hexane, hexane:ethyl acetate (9:1), and hexane:ethyl acetate (8:2). The authors concluded that carefully optimized and controlled saponification conditions can avoid vitamin E degradation and improve quantification. Our personal experiences with RSM optimization show the technique to be a highly desirable matrix-specific approach with the potential to improve extractions for most water- and fat-soluble vitamins.

3.3.3.2.1.2 Direct solvent extraction. Methods normally used for total lipid extraction from biological matrices have been used for vitamin E extraction before quantification. Some of the earliest studies on the vitamin E content of food used hot absolute ethanol. Such methods can yield an extract that can be directly injected onto the LC column without additional cleanup. Many organic solvents and solvent mixtures efficiently extract vitamin E from the sample matrix. Ball[103–106] summarized solvent requirements to include effective penetration of the sample matrix while stabilizing the vitamin E. Under most circumstances, complete liquid extraction must be assured to accomplish complete removal of vitamin E from the sample matrix. Most physiological fluids can be extracted with simple direct solvent extraction procedures. These procedures usually have the following sequence as summarized by Eitenmiller and Lee:[43]

1. Addition of a protein denaturing solvent such as isopropanol, ethanol, methanol, or acetonitrile

2. Addition of water or buffer to improve the extraction efficiency of the solvent
3. Addition of the organic phase to extract the vitamin E
4. Centrifugation
5. Solvent evaporation, if required, to concentrate the analytes

Some of the most commonly used solvents for vitamin E extraction include the Folch extraction with chloroform:methanol (2:1), acetone, diethyl ether, and Soxhlet extraction with a variety of solvents.[103] If the sample is dry, Soxhlet extraction is often the simplest and most efficient method for vitamin E extraction. Wet samples can be ground in the presence of excess anhydrous sodium sulfate to produce a dry powder suitable for Soxhlet extraction.[103] Suitable solvents include absolute ethanol, acetone, *n*-hexane, and petroleum ether. Because of the extensive time required for the extraction, an antioxidant such as BHT must be added to the extraction solvent. Protection from light is required throughout the procedure. If the sample contains polar lipids in quantity, *n*-hexane might not be sufficiently polar to efficiently extract the lipids, and the recovery of vitamin E homologs will be low.

Several investigators developed rapid one-step extractions for serum that yield an extract that can be directly injected into the LC.[243-245] Rupérez et al.[243] compared n-propanol, dichloromethane:*n*-propanol (85:15), diethylether, acetone:isopropanol (85:15), acetone: SDS (0.05 M) (85:15), acetone, SDS, and acetone:isopropanol (50:50) for the following attributes in extraction of α-T from human serum:

1. Compatibility with the mobile phase
2. Ability to precipitate plasma proteins
3. Provide a "clear and clean" extract
4. High recovery of analyte(s)

The researchers chose acetone as the best extractant for high recovery and compatibility for direct injection. Other solvents used for direct injection after a one-step extraction include n-butanol:ethyl acetate:acetonitrile (1:1:1)[244] and acetonitrile in combination with tetrahydrofuran.[245]

Hakansson et al.[166] developed a Soxhlet extraction procedure for the determination of vitamin E in wheat products. The extraction used 5–15 g of cereal ground to pass a 1.0 mm sieve. Extraction was completed with 125 mL of *n*-hexane containing 1 mg of BHT at 90°C for 4 h. After extraction, the hexane extract was evaporated to near dryness using a rotary evaporator at 50°C. The residue was redissolved with 15 mL hexane and 15 mL of 99.5% ethanol and re-evaporated. The residue was redissolved in *n*-hexane and filtered through a 0.45 mm filter before HPLC quantification. The extract could be stored in amber bottles overnight if refrigerated. The Soxhlet procedure was compared to the solvent extraction procedure of Thompson and Hatina.[167] Recoveries of α- and β-T for the Soxhlet extraction (95%–100%) exceeded those obtained by the isopropanol/acetone solvent system developed by Thompson and Hatina.[167]

Thompson and Hatina's[167] solvent extraction procedure for tocopherols and tocotrienols has been used by many investigators for vitamin E extraction from a wide array of natural products. The procedure is based on isopropanol and acetone extraction combined with partitioning of the vitamin E fraction into *n*-hexane. A summary of the procedure follows: homogenize 10 g of sample with 100 mL of boiling isopropanol in the cup of a Virtis homogenizer. After 1 min of homogenization, add 50 mL of acetone and filter the mixture through Whatman GF/A glass fiber paper into a 500 mL separatory funnel. The residue was extracted with 50 mL of acetone, and the filter paper and its contents were homogenized with 100 mL of acetone. This extract was also filtered into the separatory funnel and the residue washed with 50 mL of acetone. Add 100 mL of hexane to the pooled extracts and swirl the funnel to mix the contents. Add 100 mL of water and swirl the funnel to mix the phases. After phase

separation, the *n*-hexane epiphase was transferred to a second funnel and the aqueous hypophase was extracted two more times with 10 mL portions of *n*-hexane. The pooled *n*-hexane extracts were washed twice with 100 mL portions of water and evaporated under vacuum. The efficiency of the procedure was 97% or greater depending on the matrix being extracted.

Landen[168] used high-performance gel permeation chromatography HP-GPC and reversed-phase chromatography (RP-HPLC) after extraction to simultaneously determine all-*rac*-α-T acetate and vitamin A palmitate in infant formulas. By avoiding saponification, this HPLC method can be used to simultaneously determine all-*rac*-α-T acetate and the natural vitamin E forms present in infant formulas. Landen et al.[169] successfully applied HP-GPC and RP-HPLC techniques for the determination of all-*rac*-α-T acetate and vitamin A palmitate levels in a large number of infant formulas, representing different batches and different matrices, with further application to medical foods and other products. The extraction method uses magnesium sulfate to dehydrate the sample and extraction with isopropanol and methylene chloride. The fat was removed from the extract by gel permeation chromatography followed by quantitation on reversed-phase LC with UV detection. The method is discussed in Chapter 14. Chase et al.[170] modified Landen's extraction procedure for infant formula by eliminating methylene chloride from the extraction. The extraction was completed with isopropanol and hexane:ethyl acetate (85:15).

Modifications of Landen's extraction have shown that the HP-GPC fractionation is not necssary for vitamin E analysis since normal-phase chromatography is used almost universally, and lipid removal from the extraction solvent is not required before injection. Studies using modifications of the original method include Chase et al.,[170,171] Ye et al.,[172–174] and Lee et al.[45,175]

Lee et al.[175] specifically modified the extraction for use on peanuts, peanut butter, and other nuts and compared the direct solvent extraction to saponification for general utility. The procedure included the following steps:

1. Weigh 0.4 g sample into a 125 mL round-bottom flask.
2. Add 4 mL of hot (80°C) water.
3. Mix with a spatula.
4. Add 10 mL isopropanol, 5 g magnesium sulfate, and 25 mL of hexane:ethyl acetate (90:10) containing 0.01% BHT.
5. Homogenize for 1 min with a homogenizer (Polytron), and rinse the tip with 5 mL of extracting solvent.
6. Filter the mixture through a medium-porosity glass filter using a vacuum bell jar filtration unit.
7. Break up filter cake and wash with 5 mL extracting solvent.
8. Transfer the filter cake to the 125 mL flask and reextract with homogenization with 5 mL of isopropanol and 30 mL of extracting solvent.
9. Transfer the combined filtrates to a 100 mL volumetric flask and dilute to volume with extracting solvent.
10. Aliquots of the extract can be concentrated under N_2 if required before injection.

The procedure gave higher values for each of the vitamin E homologs when compared to other procedures. When it was coupled with normal-phase chromatography, highly reproducible results were obtained from peanuts, peanut butter, and several other nuts. Recoveries from peanut butter approached 100% for each of the vitamin E homologs. When used on a routine basis, the extraction is fast with low solvent requirements. It is also applicable to studies requiring the assay of all-*rac*-α-tocopheryl acetate together with natural vitamin E in fortified products.

Comparison of traditional saponification to newer methods of extraction for vitamin E was completed by Delgado-Zamarreño et al.[176] using nuts and seeds. Methods compared to saponification included saponification followed by continuous membrane extraction coupled to the LC and direct extraction of tocopherols through a silicone membrane coupled online with the LC. Each procedure gave good extraction results. The authors considered that the direct extraction with application of the membrane filtration offered significant potential for food analysis because of the speed and precision of the analysis. The complete assay of a sample including extraction required 40 min. The extraction would require validation before application to other matrices.

3.3.3.2.1.3 Matrix solid-phase dispersion. Matrix solid-phase dispersion (MSPD) represents a patented extraction procedure first used to isolate drugs from milk and tissue. Chase and Long[177] and Chase et al.[178,179] introduced MSPD as an extraction procedure for tocopherols, all-*rac*-α-tocopheryl acetate, and retinyl palmitate in infant formula and medical foods. The technique is based on the dispersion of the sample onto C_{18} to form a powder that is subsequently eluted with a solvent capable of eluting the analytes of interest. The stepwise procedure applied to infant formula and medical foods follows:

1. Weigh 2 g of C_{18} (Bondesil) into a mortar.
2. Add 100 μL of isopropyl palmitate and gently blend the isopropyl palmitate onto the C_{18} with a pestle.
3. Accurately weight 0.5 g of reconstituted sample (10 g of infant formula powder was mixed with 50 g of 80°C water) onto the C_{18}/isopropyl palmitate mixture.
4. Gently blend the mixture into a fluffy, slightly sticky powder.
5. Transfer the mixture into a 15 mL reservoir tube with a frit at the bottom, and insert the top frit on top of the powdery mix.
6. Tightly compress the reservoir contents with a 10 mL syringe plunger.
7. Pass 7 mL of isopropanol:hexane (0.5:99.5), followed by 7 mL of methylene chloride for infant formula or 7 mL of methylene chloride:ethyl acetate:0.5% isopropanol in hexane (3 + 3 + 4 v/v) for medical foods through the reservoir, collecting both eluents into a 50 mL vessel (Turbovap).
8. Evaporate to near-dryness at 45°C in a Turbovap under 5-psi nitrogen.
9. Dilute residue to 1.0 mL with hexane.
10. Inject.

MSPD provides an alternative to traditional saponification and direct solvent extraction procedures used for infant formula and medical foods that reduces use of organic solvents, is amenable to further automation, and is very rapid. Published studies gave low coefficients of variation and recoveries above 90% for assay of all-*rac*-α-tocopheryl acetate and retinyl palmitate. No problems were found with the assay of native tocopherols together with the synthetic fortificants. However, the presence of encapsulated vitamins can pose a problem for the method. Addition of isopropyl palmitate as a modifier was necessary for efficient elution of retinyl palmitate from the MSPD column. It was theorized that the isopropyl palmitate competes for binding sites on the C_{18}. The presence of the isopropyl palmitate in the final extract increases the viscosity of the extract and must be considered with use of autoinjectors to ensure reproducibility of injection volumes.

3.3.3.2.1.4 Pressurized liquid extraction. Pressurized liquid extraction (PLE) or accelerated solvent extraction (ASE) uses an organic solvent or a mixture of solvents at high pressure above the solvent boiling point for extraction. The method represents a modification of the Soxhlet extraction, which is more rapid and requires less volume of extraction solvent.[180] PLE has been used with good success in the extraction of tocopherols from nuts and seeds.[180,181]

Delgado-Zamarreño et al.[180] used PLE to extract tocopherols from almonds, sunflower seeds, hazelnuts, and walnuts. The extraction used a Dionex ASE 100 automated system with the following steps:

1. One gram ground sample was mixed with Hydromatrix Celite in the extraction cell.
2. The extraction used two cycles of 5 min at 1600 psi with acetonitrile.
3. The extracts were diluted to 50 mL with acetonitrile, filtered, and injected into the LC. Recoveries ranged from 82% to 110% and results closely compared to results obtained with saponification.

In a similar application, Sivakumar et al.[181] extracted α-T from hazelnut powder with hexane containing 0.01% BHT, also, with the Dionex ASE 100 unit.

3.3.3.2.2 Chromatography parameters. Since the literature on applications of LC to vitamin E analysis of foods and biologicals is extensive, selected references are provided in Table 3.9 to provide an overview of vitamin E assay methodology by LC.[182–257]

Useful reviews include Nelis et al.,[102] Rupérez et al.,[112] Eitenmiller and Lee,[43] and Ball.[106] Eitenmiller and Lee[43] summarized the literature through 2004.

3.3.3.2.2.1 Supports and mobile phases. Both reversed-phase and normal-phase LC are useful for resolution of vitamin E. Because normal-phase chromatography can resolve β- and γ-T as well as the T3 positional isomers, most studies designed to quantify all tocopherol and tocotrienol forms in food rely on normal-phase chromatography. Studies conducted with reversed-phase methods report the combined positional isomers as (β- + γ-T). Normal-phase chromatography completely resolves the eight homologs. Tan and Brzuskiewicz[94] studied various columns and mobile phases for resolution of *RRR*-tocopherols and *RRR*-tocotrienols. Normal-phase systems showed elution of the homologs in order of increasing polarity with separation based on methyl substituents on the chromanol ring. Reversed-phase systems showed class separations based on the saturation of the phytyl side-chain with the more saturated tocopherols being retained longer on the column. These authors reported optimal normal-phase chromatography on Zorbax SIL with a binary isocratic mobile phase of hexane:isopropanol (99:1). For normal-phase chromatography on Zorbax NH_2, the optimal solvent system was an isocratic mobile phase of hexane:isopropanol (99:2). Optimal reversed-phase chromatography was on Zorbax ODS with an isocratic mobile phase of acetonitrile:methanol:methylene chloride (60:35:5). Typical chromatograms for the tocopherols and tocotrienols are given in Figure 3.6. Direct injection of oils requires a normal-phase system because the triacylglycerols remain soluble in the mobile phase and do not interfere with the chromatography.

Normal-phase chromatography on silica can accommodate up to 2 mg of fat per injection.[167] Examination of the current methods presented in Table 3.9 show that normal-phase chromatography is almost universally used for quantitation of vitamin E. Gradient systems are useful for better resolution of the eight homologs. Particularly, increased resolution of γ-T and γ-T3 can be obtained with gradient chromatography. Slightly elevated column temperatures can increase resolution.

Abidi and Mounts[258] showed the effects of various mobile phase conditions on the separation of β- and γ-T using aminopropyl silica or diol-silica supports. Previous work showed the molecular polarity and steric factors of the 5-, 7-, and 8-methyls interact with the 6-hydroxy group to significantly affect resolution. The dimethyl homologs, because of close polarity, are the most difficult vitamin E homologs to resolve by normal-phase chromatography and impossible to resolve by reversed-phase chromatography. The study showed that the ability of mobile phases containing a weakly polar modifier such as an ester (ethyl acetate) or a monofunctional ether (*tert*-butyl methyl ether) was significantly greater

Table 3.9 LC and LC-MS Methods for the Analysis of Vitamin E

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods					
Oils and Fats					
Seed oils/α-, β-, γ-, δ-T, α-, β-, γ-, δ-T3	Dilution w/Hex. Direct injection	Polygosil 60-5, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic Hex:DIPE (90:10) Flow rate—1.8 mL min^{-1}	Fluorescence Ex λ = 296 Em λ = 320	QL—4 µg g^{-1} %CV—2.9–8.4 %Recovery—93–95	*J. Food Sci.*, 50, 121, 1985[159]
Vegetable oils/α-, β-, γ-, δ-T	Dilution w/Hex. Direct injection	LiChrosorb Si60, 5 µm, 4.0 mm × 25 cm, 30°C Mobile phase—gradient 8–17% DIPE in Hex Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 325	QL—0.25 mg 100 g^{-1}	*JAOCS*, 63, 328, 1986[182]
Vegetable oils/α-, β-, γ-, δ-T	Dilution w/Hex. Direct injection	LiChrosorb Si60, 5 µm, 4.6 mm × 25 cm Mobile phase—isocratic 3% dioxane in Hex Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 295 Em λ = 330	—	*J. Food Compos. Anal.*, 1, 231, 1988[183]
Rice bran oil/α-, β-, γ-, δ-T, α-, β-, γ-, δ-T3, γ-oryzanol	Dilution w/Hex. MeCN: MeOH:IPA (50:45:5). Direct injection	Hypersil ODS, 5 µm, 2.1 mm × 20 cm Mobile phase—gradient a. 0–5 min MeCN:MeOH:IPA:water (45:45:5:5) b. 5–10 min MeCN:MeOH:IPA (50:45:5) Flow rate—1 mL min^{-1}	Fluorescence (a) Vitamin E Ex λ = 290 Em λ = 320 (b) Oryzanol 325 nm	—	*JAOCS*, 70, 301, 1993[184]
Vegetable oils/α-, β-, γ-, δ-T	Add tocol (IS). Dilute w/ CH_2Cl_2. GPC cleanup, four columns in series. Collect vitamin fraction. Evaporate. Redissolve in mobile phase	Ultrasphere silica, 5 µm, 25 cm × 4.6 mm Mobile phase – isocratic Hex:IPA (99.3:0.7) Flow rate—1 mL min^{-1}	1. Evaporative light scattering (ELSD) 2. Fluorescence Ex λ = 290 Em λ = 330	DL (ng) on-column 250 (ELSD) 25 (fluorescence)	*JAOCS*, 71, 877, 1994[185]

Olive oil/α-, β-, γ-, δ-T, α-, β-, γ-, δ-T3	(a) NP-HPLC. Dilute w/ Hex. Direct injection. (b) RP-HPLC. Dilute w/THF. Dilute w/ MeOH. Direct injection	(a) LiChrosorb Si60, 5 μm, 25 cm × 4 mm (b) Spherisorb ODS, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic NP-HPLC, Hex:IPA (99.7:0.3) Flow rate—1.7 mL min^{-1} RP-HPLC, 0.05 M $NaClO_4$: MeOH (10:90) Flow rate—2 mL min^{-1}	NP-HPLC (a) Fluorescence Ex λ = 290 Em λ = 330 (b) PDA 280 nm RP-HPLC Amperometric—0.6 V	QL—1.9 mg 100 g^{-1} (γ-T3) $\%RSD_R$ α-T—0.5 α-T3—0.9 β-T3—6.8 γ-T3—1.5 δ-T3—5.3	*JAOCS*, 72, 1505, 1995[186]
Rapeseed oil w/wo added antioxidants/ α-, β-, γ-, δ-T	Dilute w/Hex. Direct injection	Apex Silica, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic Hex:IPA (98.5:1.5) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 330	—	*Food Chem.*, 52, 175, 1995[187]
Vegetable oils/α-, (β- + γ-), δ-T	Dilute w/Hex. Direct injection	ODS-2, 5 μm, 15 cm × 4.0 mm Mobile phase—isocratic MeOH:water (96:4) Flow rate—2 mL min^{-1}	PDA 292 nm	DL (ng) on-column α-T—11.5 δ-T—12 QL (ng) on-column α-T—23 δ-T—25 %Recovery >92	*J. Chromatogr. A*, 881, 251, 2000[188]
Olive oil/α-T	Saponification, 70°C, 30 min. Extract w/Hex:EtOAC (85:15). Evaporate. Redissolve in MeOH	ODS-2, 5 μm, 15 cm × 4.0 mm	PDA 292 nm	DL (ng) on-column α-T—11.5 β-carotene—15.5 QL (ng) α-T—23 β-carotene—31 %Recovery—85	*J. Chromatogr. A*, 881, 255, 2000[189]

Continued

Table 3.9 (Continued)

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Oils and Fats (*Continued*)					
Vegetable oil/α-, γ-, δ-T	74 g oil + 6 g Triton ×–114, 10 g MeOH, 10 g Hex	OD-224 RP-18, 5 μm, 4.6 × 220 mm Mobile phase—isocratic 2.5 mM HAC/NaOAC in MeOH:water (97:3) Flow rate—1 mL min^{-1}	EC a. Porous graphite +1000 mV b. Reference +500 mV IS-PMC	%RSD α-T—6.65 γ-T—6.35 δ-T—6.35	*J. Chromatogr. A*, 881, 229, 2000[190]
Olive oil/α-, β-T	Dissolve in MeOH:IPA (80:20). Evaporate, 40°C. Redissolve in MeOH:IPA:Hex (1:3:1)	Apex octadecyl C_{18}, 5 μm, 25 cm × 4.0 mm Mobile phase – gradient A—HAC (2%) B—MeOH C—MeCN D—IPA	280 nm	%Recovery—79–87	*Food Chem.*, 74, 327, 2001[191]
Shea butter/α-, β-, γ-, δ-T	Extract fat w/Hex. Direct injection	Spherisorb SS NH_2, 5 μm, 25 cm × 4 mm Mobile phase—isocratic Hex:EtOAC (75:25) Flow rate—1 mL min^{-1}	265 nm	—	*J. Agric. Food Chem.*, 52, 2934, 2004[192]
Palm oil/α-, β-, γ-T, α-, γ-, δ-T3, α-tocomonoenol	Direct injection	Develosil RP C_{30} Aqueous, 25 cm × 4.6 mm Mobile phase—isocratic MeOH—100% Flow rate—1 mL min^{-1}	PDA MS NMR	—	*Lipids*, 39, 1031, 2004[193]
Cannabis sativa L. oil/α-, β-, γ-, δ-T, plasto-chromanol-8	SFE—CO_2. Dilute w/Hep. Direct injection	Diol phase, 25 cm × 4.6 mm Mobile phase—isocratic Hep:TBME (99:1) Flow rate—1.3 mL min^{-1}	Fluorescence Ex λ = 295 Em λ = 330	—	*Euphytice*, 137, 339, 2004[194]
Date palm seed oil	Soxhlex—Hex. Dilute w/Hex. Direct injection	LiChrosorb Si60, 5 μm, 25 cm × 4.6 mm Mobile phase – isocratic Hex:IPA (98.5:1.5) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 330	—	*J. Food Lipids*, 11, 251, 2004[195]

Sudanese oils/α-, γ-, δ-T	Various solvents. Direct injection	Diol phase, 25 cm × 4.6 mm Mobile phase—isocratic Hep: TBME (99:1) Flow rate—1.3 mL min^{-1}	Fluorescence Ex λ = 295 Em λ = 330	—	*J. Food Lipids*, 11, 179, 2004[196]
Vegetable oils/α-, γ-, δ-T, α-TAC	Saponification, 60°C, 45 min	Luna C_8, 3 μm, 15 cm × 2 mm Mobile phase—isocratic MeCN:water (95:5) Flow rate—0.4 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 325	DL (ppm) α-T—6.1 γ-T—0.76 δ-T—0.41	*J. Food Compos. Anal.*, 19, 141, 2006[197]
LC-MS					
Palm oil extract/ α-T, α-, β-, γ-, δ-T3, α-tocoenol	Dissolve extract in EtOH	YMC-gel C_{30}, 3 μm, 200 Å, 25 cm × 4.6 mm	MS-CIS	—	*Anal. Chem.*, 71, 1780, 1999.[224]
Margarine, Mayonnaise Peanut Butter					
Full-fat margarine/α-T	Dissolve in Hex. Direct injection	Hypersil, 5 μm, 10 cm × 2.1 mm Mobile phase—isocratic Hex:IPA (99.8:0.2) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 330	%Recovery >90	*J. High Resolut. Chromatogr.*, 16, 536, 1993[198]
Full and reduced fat margarine/α-T	Saponification, 60 mm. Extract w/PE	μBondapak C_{18}, 10 μm, 25 cm × 4 mm Mobile phase—isocratic MeOH:water (93:7) or (92:8) Flow rate—1.5 mL min^{-1}	280 nm	%Recovery—94.3 ± 7.4	*Food Chem.*, 58, 373, 1997[199]
Full fat and reduced fat margarine, peanut butter/α-, γ-, δ-T	To sample, add hot water (80°C), IPA, $MgSO_4$, followed by Hex:EtOAC (90:10) or (85:15) containing 0.01% BHT, homogenize, filter. Reextract filter cake. Dilute to volume w/Hex:EtOAC	LiChrosorb Si60, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic Hex:IPA (99.1:0.9) Flow rate—1 mL min^{-1} or Gradient flow rate—0.9–1.5 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 330	%Recovery > 90 for α-, β-, γ-, δ-T	*J. Liq. Chromatogr. Rel. Technol.*, 21, 1227, 1998;[172] *J. Agric. Food Chem.*, 48, 4003, 2000;[173] *J. Food Sci.*, 65, 1, 2000;[174] *Peanut Sci.*, 25, 123, 1999;[175] *J. Food Sci.*, 68, 2211, 2003;[200] *Food Sci. Biotechnol.*, 15, 183, 2006.[45]

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Table 3.9 (Continued)

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Infant Formula, Milk, Medical Foods					
Infant formula/α-TAC	Extract w/dimethylsulfoxide: dimethylformamide: $CHCl_3$ (200:200:100). Partition w/Hex. Clarify Hex layer by centrifugation	Rad-Pak silica cartridge, 5 µm, 8 mm i.d. Z-module compression unit Mobile phase—isocratic Hex:IPA (99.92:0.08) Flow rate—2 mL min^{-1}	280 nm	QL—0.7 IU 100 g^{-1} %Recovery—92–93	*J. Micronutr. Anal.*, 2, 97, 1986;[201] *J. Micronutr. Anal.*, 3, 1, 1987[202]
Human milk, infant formula/α-, β-, γ-, δ-T, α-, β-, γ, δ-T3	Saponification, ambient, overnight	LiChrosorb Si60, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic Hex:DIPE (93:7) Flow rate—2.5 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 325	%Recovery—80–90 %CV—3.8–7.2	*J. Vit. Nutr. Res.*, 55, 159, 1985[148]
Infant formula/α-, β-, γ-, δ-T	Extract fat by the Ross-Gottlieb procedure. Saponify lipid fraction. Extract w/Hex. Evaporate. Redissolve in IPA:EtOH:Hex (1:0.5:98.5). Saponify lipid fraction	LiChrosorb Si60, 5 µm, 12 cm × 4.6 mm Mobile phase—isocratic IPA:EtOH:Hex Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 292 Em λ = 320	%Recovery—96–108	*J. Micronutr. Anal.*, 6, 35, 1989[156]
Infant formula, milk, various foods/α-, β-, γ-, δ-T	Saponify. Extract w/light petroleum:DIPE (2:1). Centrifuge. Inject 10 µL of upper layer	Rad-Pak silica, 5 µm, RCM-100 Mobile phase—isocratic Hex:IPA (99:1) Flow rate—1 mL min^{-1} Rad-Pak C_{18}, 5 µm, RCM-100 Mobile phase—isocratic 100% MeOH Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 295 Em λ = 330	QL—0.4 mg 100 g^{-1} %Recovery—93–97 %CV (within run)—1.9–5.7	*Analyst*, 113, 1217, 1988[152]
Infant formula/α-T	Saponify, 70°C, 25 min. Extract w/Hex:CH_2Cl_2 (3 + 1). Evaporate. Redissolve in mobile phase. Hex:IPA (99.92:0.08).	Hypersil silica, 5 µm, 25 cm × 4.6 nm Mobile phase—isocratic Hex:IPA (99.92:0.08) Flow rate—1 mL min^{-1}	280 nm	—	*J. AOAC Int.*, 76, 399, 1993[119]

Human milk/α-, γ-T	Add α-T acetate (IS). Dilute w/EtOH. Extract w/Hex. Evaporate. Dissolve in Hex containing 0.1% BHT	Rad-Pak silica, 5 μm, 8 mm i.d Mobile phase—isocratic Hex:DIPE (95:5) Flow rate—2.5 mL min^{-1}	280 nm	—	*Nutr. Res.*, 6, 849, 1986[203]
Infant formula/α-T	Interlaboratory study. Various methods.	Various	Fluorescence 280 nm	%RSD_R—16	*Analyst*, 118, 475, 1993[204]
Infant formula/α-, β-, γ-, δ-T, α-TAC	Extract w/CH_2Cl_2:MeOH (2:1). Add H_2O. Shake. Remove CH_2Cl_2 phase. Dry under N_2	Nova-Pak Silica, 5 μm, 15 cm × 3.9 mm Mobile phase—isocratic Hex:EtOAC (98:2) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 295 Em λ = 330	%RSD—7–9.1	*J. Chromatogr. A*, 947, 97, 2002[205]
Human milk/α-, γ-, δ-T	Spike w/δ-T (IS). Saponify, 30 min, 70°C. Acidify to pH 2.0. Extract w/Hex. Or add MeOH; extract w/Hex	Waters Symmetry C_{18}, 3 μm, 15 cm × 3.9 mm Mobile phase—gradient MeCN in water to MeCN Flow rate—1 mL min^{-1}	PDA 275–350 nm	DL—0.65 μg mL^{-1} for δ-T	*Acta Chromatogr.*, 16, 48, 2006[164]
LC-MS					
Infant formula, cereal foods/α-T	Saponification. Add d_6-RRR-α-TAC (IS). Digest w/ Taka-Diastase, 30 min, 45°C. Saponify, 30 min, 70°C. Extract w/benzine: DIPE (75:25)	Spherisorb Si, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic Hex:IPA (99:1) Flow rate—1 mL min^{-1}	APCI-MS Positive ion mode/ SIM	DL—2.5 ng mL^{-1} %CV <3.5	*Rapid Commun. Mass Spectrom.*, 17, 723, 2003[206]
Cereals					
Cereal products/α-, β-, γ-, δ-T, α-, β-, γ-, δ-T3	Saponification, ambient, overnight	LiChrosorb Si60, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic Hex:DIPE (93:7) Flow rate—2.1–2.5 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 325	%Recovery—80–99	*Cereal Chem.*, 63, 78, 1986[207]
Wheat products/α-, β-, γ-, δ-T, α-, β-, γ-, δ-T3	Extract w/Hex containing BHT, 4 h, 90°C. Evaporate. Redissolve in Hex:EtOH (1:1). Evaporate. Redissolve in Hex	Chrompack LiChrosorb Si60, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic Hex:DIPE (93:7 or 97:3) Flow rate—2.5 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 330	%Recovery—86.4–100.1	*J. Micronutr. Anal.*, 3, 307, 1987[166]

Continued

Table 3.9 (Continued)

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Cereals (*Continued*)					
Grain amaranths/α-, β-, γ-, δ-T, α-, β-, γ-, δ-T3	Extract w/ MeOH. Evaporate. Extract w/Hex	Waters silica, 5 μm, 30 cm × 4.6 mm Mobile phase—isocratic Hex:IPA (99.8:0.2) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 295 Em λ = 330	—	*Lipids*, 29, 177, 1994[208]
Maize kernels/α-, γ-, δ-T	Add δ-T (IS). Saponify, 10 min, 85°C. Extract w/Hex	Nova-Pak C_{18}, 4 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:MeOH:CH_2Cl_2 (75:20:5) containing 0.05% TMA Flow rate—1 mL min^{-1} or Mobile phase—gradient Linear gradient, 0–10% CH_2Cl_2 w/3% MeOH Flow rate—1.5 mL min^{-1}	PDA 265–500 nm	—	*Phytochem. Anal.*, 11, 370, 2000[209]
Rice bran/α-, β-, γ-, δ-T, α-, β-, γ-, δ-T3	Extract w/Hex. Evaporate. Redissolve in Hex	Waters silica, 5 μm, 30 cm × 4.6 mm Mobile phase—isocratic Hex:IPA (99.8:0.2) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 295 Em λ = 330	—	*J. Agric. Food Chem.*, 48, 3130, 2000[210]
Cereals/α-, β-, γ-, δ-T, α-, β-, γ-, δ-T3	Saponification. Extract 2× w/Hex:EtOAC (9:1)	Kromasil Phenomenex Si, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic Hex:EtOAC:HAC (97.3:1.8:0.9) Flow rate—1.6 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 330	%Recovery >95	*J. Agric. Food Chem.*, 51, 3940, 2003[211]
Rye flours/α-, β-, γ-, δ-T, α-, β-, γ-, δ-T3	Saponification. Extract w/ Hex:EtOAC (8:2)	Intersil Si, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic Hex:1,4 dioxane (97:3) Flow rate—2 mL min^{-1}	Fluorescence Ex λ = 292 Em λ = 325	%Recovery—90.3–94.3	*J. Food Compos. Anal.*, 17, 749, 2004[160]

Rice bran/α-, γ-, δ-T, α-, γ-, δ-T3	Direct solvent extraction. MeOH at ratio of rice bran to MeOH of 1:60	Nova-Pak C_{18}, 4 μm, 15 cm × 3.9 mm Mobile phase—gradient MeCN:MeOH:IPA: HAC (45:40:5:10) 3 min To (45:45:5:5) over 1 min To MeCN:MeOH:IPA (25:70:5) over 10 min (hold 11 min)	Fluorescence Ex λ = 298 Em λ = 328	%Recovery—92–102	*J. Food Compos. Anal.*, 18, 139, 2005[212]
LC-MS					
Wheat bran/α-, γ-, δ-T	Direct solvent extraction 50% A	Zorbax SB C_{18}, 3.5 μm, 3 cm × 1 mm Mobile phase—gradient Solvent A—water Solvent B—MeCN 80–90% B Flow rate—0.3 mL min^{-1}	LC-ESI-MS/MS SRM monitoring Positive-ion mode	—	*J. Agric. Food Chem.*, 53, 3916, 2005[213]
Corn fiber/α-, β-, γ-, δ-T, α-, β-, γ-, δ-T3	Extract w/Hex	DIOL, 7 μm, 10 cm × 3 mm Mobile phase—gradient Solvent A—Hex:THF (98:2) Solvent B—IPA 100% A—40 min To 95% A—5 min To 100 % A—1 min Flow rate—0.5 mL min^{-1}	Fluorescence Ex λ = 294 Em λ = 326 LC-APCI-MS Positive-ion mode	—	*J. Agric. Food Chem.*, 54, 8093, 2006[214]
Nuts and seeds					
Pecans, peanuts/α-, β-, γ-, δ-T	Soxhlet. Hex containing 0.01% BHT, 90°C, 6 h. Evaporate. Redissolve in Hex containing 0.01% BHT	LiChrosorb Si60, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic Hex:IPA (99:1) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 330	DL (ng) on-column α-T—2 β-T—1 γ-T—2 δ-T—0.6 %Recovery—84–96	*J. Food Sci.*, 57, 1194, 1992;[215] *Peanut Sci.*, 20, 21, 1993;[216] *JAOCS*, 70, 633, 1993[217]

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Table 3.9 (Continued)

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Nuts and seeds (*Continued*)					
Peanuts/α-, β-, γ-, δ-T	Extract w/Hex:EtOAC (90:10) containing 0.01% BHT. Remove water w/$MgSO_4$. Evaporate. Dissolve w/Hex	LiChrosorb Si60, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic Hex:IPA (99.4:0.6) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 330	DL (ng) on-column α-T—0.2 β-T—0.1 γ-T—0.1 δ-T—0.1 %Recovery—97–102	*Peanut Sci.*, 25, 123, 1999;[175] *J. Food Sci.*, 70, C292, 2005[218]
Seeds, nuts/α-, (β- + γ-), δ-T	Saponification coupled w/continuous membrane extraction. Direct extraction through a silicone membrane or PLE	OD224 RP18, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic 2.5 mM HAC-NaOAC in MeOH:water (97:3) or (99.9:0.1) w/the Synergy Hydro RP column	EC Dual porous graphite working electrodes IS-PMC	Continuous, w/out saponification $\%RSD_{Intra}$—2.9–4.3 $\%RSD_{Inter}$—7.5–9.1 %Recovery—97–102	*J. Chromatogr. A*, 935, 77, 2001;[176] *J. Chromatogr. A*, 1056, 249, 2004[180]
Coffee beans/α-, β-, γ-T	Extract oil by Soxhlet using Hex. Dissolve in A	Superspher 100 RP-18, 4 μm, 25 cm × 4 mm Mobile phase—isocratic MeCN:A (1:1) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 330	%Recovery >89	*Food Chem.*, 73, 93, 2001[219]
Soybeans/α-, γ-, δ-T	Extract w/Hex	AOCS Official Method Ce-8-89	292 nm	—	*Crop Sci.*, 44, 772, 2004[220]
Soybeans/α-, γ-, δ-T	Sonicate ground seed in 80% EtOH. Extract w/Hex	Intersil ODS-3, 25 cm × 3 μm Mobile phase—isocratic MeCN:MeOH (75:25) Flow rate—0.5 mL min^{-1}	295 nm	—	*Breeding Sci.*, 55, 123, 2005[221]
Walnuts/α-, β-, γ-, δ-T, γ-T3	Extract w/Hex after addition of EtOH, NaCl	Intersil 5, 25 cm × 3 mm Mobile phase—isocratic Hex:1,4 dioxane (96.5:3.5) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 330	—	*J. Agric. Food Chem.*, 53, 5467, 2005[222]
Hazelnuts/α-, β-, γ-, δ-T, α-, β-, γ-, δ-T3	Homogenize in EtOH. Extract w/Hex	Intersil 5 Si, 25 cm × 3 mm Mobile phase—isocratic (95.5:4.5) Flow rate—0.7 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 330	DL—0.01-0.3 μg mL^{-1} QL—0.02–0.83 μg mL^{-1}	*Anal. Sci.*, 21, 1545, 2005[223]

Miscellaneous Foods					
Forty foods/α-, β-, γ-, δ-T	Saponification, overnight, ambient. Extract w/Hex	Zorbax ODS, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:$MeCl_2$: MeOH (70:30:5) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 330	DL—0.1 μg 100 g^{-1}	*J. Food Compos. Anal.*, 2, 200, 1989[150]
Chicken muscle/ α-, δ-T	Saponification, overnight, ambient. Extract w/Hex	Biosil ODS-55, 25 cm × 4.6 mm Mobile phase—isocratic MeOH—100% Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 296 Em λ = 330	%Recovery—92–93	*J. Food Sci.*, 55, 1536, 1990.[225]
Multivitamins, foods/ α-, β-, γ-, δ-T, α-TAC, plastochromanol-8	Add water, EtOH, shake, and sonicate. Add tBME, centrifuge. Repeat 2×. Add EtOH. Evaporate	LiChrospher 100 DIOL, 5 μm, 250 cm × 4.6 mm Mobile phase—gradient Hex:tBME 100% Hex to Hex:tBME (95:5) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 280 Em λ = 335 or Ex λ = 295 Em λ = 330	%Recovery—100–103 DL (on-column)—2.2–4.6 ng for α-TAC	*Fat Sci. Technol.*, 95, 215, 1993[226]
Animal diets/α-T, α-TAC	Sonicate in EtOH:MeOH: water (1:1:1.5)	Nucleosil 120 C_{18}, 5 μm, 15 cm × 4.6 mm Mobile phase—isocratic MeOH:water (94:6) Flow rate—2 mL min^{-1}	Fluorescence α-T Ex λ = 295 Em λ = 350 UV α-TAC 284 nm	%Recovery > 85 %RSD <6	*J. Chromatogr. A*, 839, 93, 1999[227]
Tomato, broccoli/ α-, γ-T	Saponification—optimized, 70°C, 50.7–54.3 min. EtOH—30.1–35%	LiChrosorb Si60, 5 μm, 25 cm × 4.0 mm Mobile phase—isocratic Hex:IPA (99.1:0.9) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 330	$\%RSD_{Intra}$—3.18 $\%RSD_{Inter}$—3.71	*J. Food Compos. Anal.*, 13, 45, 2000[157]

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Table 3.9 (Continued)

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Miscellaneous Foods (*Continued*)					
Bulgarian foods/α-T	Saponification. Extract w/ Et_2O	LiChrosorb C_{18}, 25 cm × 4.6 mm Mobile phase—isocratic 100% MeOH Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 285 Em λ = 345	—	*J. Food Compos. Anal.*, 16, 659, 2003[228]
Olive-based foods/ α-, γ-T	Homogenize w/water, A containing 0.1% BHT (repeat 3–4×). Add Et_2O to pooled extracts. Add water. Separate Et_2O phase. Evaporate aliquot	Spherisorb W silica, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic Hex:IPA (99.5:0.5) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 330	—	*JAOCS*, 82, 129, 2005[229]
Fruits, vegetables/ α-, β-, γ-, δ-T, α-, β-, γ-, δ-T3	Saponification, 70°C, 30 min	LiChrosorb Si60, 5 μm, 25 cm × 4 mm Mobile phase—isocratic Hex:IPA (99.1:0.9) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 285 Em λ = 325	%Recovery 93.6–96.8 for fruits 94.8–97.8 for vegetables	*J. Food Compos. Anal.*, 19, 196, 2006[44]
Biologicals					
Plasma/α-, (β- + γ-), δ-T	Add δ-T in MeCN (IS). Extract w/*n*-butanol:EtOAC: MeCN (1:1:1). Centrifuge. Inject organic phase	LiChrocart Superspher 100 RP-18, 4 μm, 25 cm × 4 mm Mobile phase—isocratic EtOH:MeOH (90:10) at 2.5 mM $HClO_4$ and 7.5 mM $NaClO_4$ Flow rate—0.6 mL min^{-1}	EC +0.35 V	DL (on-column)—60 p. for α-T	*J. Chromatogr.*, 620, 268, 1993[230]
Rat blood, tissue/α-T stereoisomers	Add PMC, NaCl, ethanolic pyrogallol. Saponify, 70°C, 60 min. Extract w/Hex: EtOAC (90:10). Collect Hex layer. Evaporate	Chiral Pak OP (+), 25 cm × 4.6 mm Mobile phase—isocratic MeOH:water (96:4) Flow rate—0.3 mL min^{-1}	284 nm	%Recovery—91–100	*J. Nutr. Sci. Vitaminol.*, 39, 207, 1993[231]

Plasma, erythrocytes/α-T	Add α-tocopheryl acetate (IS) in EtOH. Extract w/Hex. Centrifuge. Transfer Hex phase. Evaporate. Redissolve in MeOH:Et_2O (75:25)	Spherisorb ODS-2, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic 100% MeOH Flow rate—1 mL min^{-1}	PDA 280–400 nm	DL—5.18 mmol L^{-1} QL—7.56 mmol L^{-1}	*J. Chromatogr. B*, 660, 395, 1994[232]
Plasma, human, rat tissue/α-T	Add water, EtOH and menaquinone-8 (IS). Extract w/Hex. Centrifuge. Evaporate Hex. Redissolve in EtOH	Capsell Pak C87, 5 μm, 15 cm × 4.6 mm Mobile phase—isocratic EtOH:MeOH:water (8.2:0.8:1.0) containing 0.05 M $NaClO_4$ Flow rate—0.8 mL min^{-1}	EC +0.6 V versus Ag/AgCl	DL (on-column)—20 pg for α-T	*Biol. Pharm. Bull.*, 17, 997, 1994[233]
Plasma/α-T, α-TAC	Add MeOH. Centrifuge. Filter. Direct injection	LiChrosorb RP-18, 10 μm, 20 cm × 4.6 mm Mobile phase—isocratic 100% MeOH Flow rate—1 mL min^{-1}	292 nm	QL (μg mL^{-1}) α-T—0.59 α-TAC—0.17	*J. Liq. Chromatogr.*, 18, 1251, 1995[234]
Liver tissue/α- (β- + γ-), δ-T	Homogenize 0.2–0.8 g in A w/0.1–1.0 mL 50 mM AA in MeOH. Vortex. Centrifuge. Filter	Spherisob ODS-2, 3 μm, 10 cm × 4.6 mm Mobile phase—isocratic MeOH:water (95:5) Flow rate—1.5 mL min^{-1}	290 nm Fluorescence Ex λ = 290 Em λ = 330	%Recovery—98 for α-T	*Comp. Biochem. Physiol.*, 113B, 143, 1996[235]
Plasma/α-T	Add 20 mL Na tungstate: $MgCl_2$ (0.06:1 M) and 1 mL MeOH to 100 mL. Centrifuge	Kromasil C_{18}, 5 μm, 10 cm × 4.6 mm Mobile phase—isocratic MeOH:MeCN:water (50:35:15) Flow rate—1.5 mL min^{-1}	292 nm	%Recovery—98	*J. Chromatogr. B*, 690, 355, 1997[236]
Tissue/α-, β-, γ-T, α-, β-, γ-T3	Pulverize w/dry ice. Add 2 mL cold water, 5 mL absolute EtOH, 4 μg of a specific tocopherol (IS). Sonicate. Add 10 mL Hex, vortex, centrifuge. Evaporate 5 mL Hex layer. Dissolve residue in Hex	Supelcosil LC-Diol, 5 μm Mobile phase—isocratic Hex:IPA (99:1) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 296 Em λ = 330	%Recovery—95	*Lipids*, 32, 323, 1997[237]

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Table 3.9 (Continued)

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Biologicals (*Continued*)					
Serum/α-T	Add EtOH. Extract w/Hex. Evaporate. Redissolve in MeOH	μ-Bondapak, C_{18}, 10 μm, 30 cm × 3.9 mm Mobile phase—isocratic MeOH:water (95:5) Flow rate—2 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 325	Assay of NIST SRM 9686	*J. Agric. Food Chem.*, 46, 973, 1998[238]
Rat plasma, tissue/ RRR- and SRR-α-T	Saponification, 70°C, 30 min. Extract w/Hex:EtOAC (90:10)	Chiraled OD-H, 25 cm × 4.5 mm in series w/sumichiral OA 4100 Mobile phase—isocratic Hex:IPA (97.3:2.7) Flow rate—0.3 mL min^{-1}	268 nm	%Recovery > 95	*Lipids*, 34, 415, 1999[239]
Plasma/α-T, d_6-α-T	Extract triacylglycerol-rich lipoprotein fraction w/Hex containing BHT (350 mg L^{-1})	Two Nucleosil EC columns in series, 25 cm × 4.6 mm, 5 μm, 100 Å Mobile phase—isocratic Hex:IPA (659.3:0.7)	297 nm	d_6-α-T DL—34 pmol mL^{-1} QL—83 pmol mL^{-1}	*J. Chromatogr. B*, 794, 1, 2003[240]
Plasma, urine/α-, γ-T, α-, γ-CEHC	Add EtOH, phosphate buffer, tocol (IS). Saponify, 70°C, 30 min. Extract w/Hex	IRIKA QC pack, 25 cm × 4 mm Mobile phase—isocratic MeOH:water:$NaClO_4$ (1000:2:7) Flow rate—1 mL min^{-1}	EC +700 mV	α-, γ-CEHC %Recovery > 93	*Eur. J. Clin. Nutr.*, 57, 410, 2003[241]
Human epidermis/α-, γ-T, α-, γ-T3	Homogenize skin in chelexed phosphate buffer containing 1 mM EDTA, BHT, SDS. Add EtOH. Extract w/ Hex	Microsorb. μMVC_{18}, 12 cm × 3 μm, 100 Å	EC +500 mV	—	*Free Rad. Biol. Med.*, 34, 330, 2003[242]

Rat plasma/α-T	Extract w/A at a ratio of A: plasma (4:1)	Supelco Discovery C_{18} Mobile phase—isocratic EC detection MeOH:A (95:5) containing 50 mM acetate buffer and 150 mM $LiClO_4$ Fluorescence and UV detection MeOH:water (95:5) Flow rate—2 mL min^{-1}	EC Glassy carbon vs. Ag/AgCl, 700 mV Fluorescence Ex λ = 295 Em λ = 350 UV 295 nm	%Recovery >94 %RSD Accuracy—5–12 Precision—1.3–6	*J. Chromatogr. B*, 800, 225, 2004[243]
Plasma/α-T	Deproteinize w/MeCN: THF (3:2). Centrifuge. Inject supernatant	Crestpak C185, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic 6% THF in MeOH Flow rate—1.5 mL min^{-1}	292 nm	%Recovery—80.7–97.6 $\%CV_{Inter}$—1.5–6.3 $\%CV_{Intra}$—1.4–3.1	*J. Chromatogr. B*, 732, 227, 1999[244]
LC-MS					
Blood components/ α-T	Add d_3-α-T (IS). Add EtOH and SDS to precipitate protein. Extract w/Hex containing BHT or saponify	Symmetry C_{18}, 3.5 μm, 5 cm × 2.1 mm Mobile phase—isocratic 100% MeOH Flow rate—0.3 mL min^{-1}	MS-ESI TOF, negative ion mode	%Recovery—98	*Rap. Commun. Mass Spectrom.*, 17, 2797, 2003[245]
Plasma/α-T, RRR-α-5-(CD_3)-T, all-*rac*-α-5,7-$(CD_3)_2$-T	Add d_3-ambo-α-T (IS). Mix 100 μL plasma w/BHT (1 mg mL^{-1} EtOH), SDS (10% in water) and EtOH. Extract w/Hex. Evaporate. Resuspend in MeOH:EtOH (1:1)	Supercosil LC18, 3 μm, 7.5 cm × 4.6 mm Mobile phase—isocratic 100% MeCN Flow rate—1 mL min^{-1}	MS/MS-APCI Positive ion w/ MRM	DL—10 pmol	*Anal. Biochem.*, 289, 89, 2001[246]
Plasma, liver, kidney/α-T, α-tocopherylquinone	Add d_3-α-T and d_6-α-tocopherylquinone (IS). Extract w/Hex after SDS treatment. Evaporate, redissolve residue in EtOH	Vydac C-18, 15 cm × 2.1 mm Mobile phase—isocratic MeCN:HAC (97:3) Flow rate—0.3 mL min^{-1}	MS/MS-APCI Positive ion mode	—	*Free Rad. Biol. Med.*, 30, 1089, 2001[247]
Gingival tissue/α-, β-, γ-, δ-T, α-TAC	Grind gingival tissue w/ 500 mg C_{18} MSPD-material. Load on SPE column. Condition w/ 5 mL water. Extract w/6 mL MeOH	Prontosil RP C_{30}, 3 μm, 120 Å, 25 cm × 4.6 mm Mobile phase—isocratic 100% MeOH Flow rate—1 mL min^{-1}	MS-APCI Positive ion mode	—	*Anal. Chem.*, 74, 5192, 2002[248]

Continued

Table 3.9 (Continued)

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
LC-MS (*Continued*)					
Plasma/α-T, α-tocopherolquinone	Add deuterated standards (IS). Saponify, 60°C, 30 min. Extraction w/Hex	Waters RP8, 3.5 μm, 15 cm × 2.1 mm Mobile phase—gradient Solvent A—water (0.1% formic acid) Solvent B—MeCN (0.1% formic acid) Linear gradient 25% A to 10% A over 20 min Back to 25% A in 4 min	MS/MS-ESI Positive ion mode w/ MRM	Accuracy > 95% QL—3 nM	*Anal. Biochem.*, 301, 128, 2002[140]
Human skin/α-, γ-T, d_3-, d_6-, d_9-α-T	Add d_9-α-T (IS). Saponify. Extract w/Hex	Supelcosil LC-18, 3 μm, 7.5 cm × 4.6 mm Mobile phase—isocratic 100% MeOH Flow rate—1 mL min^{-1}	MS-APCI Positive ion mode w/ SIR	%CV—6–7 %Recovery—94–100	*Free Rad. Biol. Med.*, 36, 456, 2004[249]
Rat liver/α-, γ-T, carboxyethyl hydrox-chromans (α-, γ-CEHC)	Add trolox (IS), 10 μL of 1% AA. Hydrolyze w/β-glucuronidase, 37°C, 30 min. Acidify. Extract w/ Et_2O. Resuspend in MeOH:water (1:1) containing 0.05% HAC and 0.05% AA	Symmetry Shield RP-18, 3.5 μm, 15 cm × 3 mm	MS-ESI Negative ion mode	%Recovery—76 for α-CEHC and 87 for γ-CEHC	*J. Lip. Res.*, 46, 1068, 2005[250]
Plasma/α-T, α-tocopheryloxyacetic acid (α-TEA)	Add 1.2 N HCL, vitamin E succinate (VES) (IS), EtOAC/A (60:40). Extract w/Hex. Centrifuge. Direct infusion into mass spectrometer	—	MS/MS-ESI [M + Ag] adducts Positive ion mode TOF	QL—0.5 μg mL^{-1} for α-T and 1.25 μg mL^{-1} for α-TEA	*Rap. Commun. Mass Spectr.*, 19, 2337, 2005[251]
Pharmaceuticals, Botanicals					
Emulsified nutritionals/α-TAC	Dissolve in 5% sodium sulfate containing 1 mM EDTA. SPE—Bond Elut C_{18}. Elute w/5%, 25%, 80% MeCN, sequentially. Elute α-TAC w/ MeCN (100%)	Intersil ODS-2, 5 μm, 15 cm × 4.6 mm Mobile phase—isocratic MeOH:EtOH (1:1) Flow rate—0.4 mL min^{-1}	Fluorescence Ex λ = 295 Em λ = 325	%Recovery > 90	*J. Chromatogr. A*, 881, 243, 2000[252]

Rosemary officinalis/α-T	Grind dry leaves. Add vitamin D_2 (IS). Add A. Sonicate. Centrifuge. Inject	Nucleosil 120 C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient Solvent A—water Solvent B MeOH:MeCN (30:70) containing 0.1% HAC 85% B to 100% over 23 min Flow rate—2.0 mL min^{-1}	PDA	%Recovery—93 $\%RSD_{Intra}$—4 $\%RSD_{Inter}$—7	*J. Chromatogr. A*, 919, 305, 2001[253]
Globularia alypum L./α-T	Extract dry leaves w/Hex	Hichrom 5 C_{18}, 25 cm × 4.6 mm Mobile phase—isocratic 100% MeOH Flow rate – 2 mL min^{-1}	292 nm	—	*Pharm. Biol.*, 42, 519, 2004[254]
Sea Buckthorn oil/α-T	Extract capsule contents w/Hex	Alltima C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:MeCN (95:5) Flow rate—1 mL min^{-1}	292 nm	%Recovery—95.8	*Chem. Pharm. Bull.*, 52, 150, 2004[255]
Laurus nobilis (bay) leaves/α-, γ-T	Add tocol (IS) to dried leaves. Add A. Sonicate w/probe. Filter. Inject	Discovery C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient Solvent A—water Solvent B MeCN:MeOH (70:30) containing 0.1% HAC 85% B to 100% B over 24 min Flow rate—2.0 mL min^{-1}	Fluorescence Ex λ = 295 EM λ = 350	%Recovery α-T—102 ± 1 γ-T—105 ± 3 %RSD α-T—1.8 γ-T—3.1	*J. Agric. Food Chem.*, 51, 5196, 2003[256]
LC-MS					
Phytopharmaceuticals/α-, β-, γ-, δ-T, α-, β-, γ-, δ-T3, α-TAC, α-T nicotinate	Food matrices Saponification, overnight, ambient. Extract w/Hex Latex milk Extract w/$MeCl_2$ and MeOH	Nucleosil 50-5, 5 μm, 50 Å, 25 cm 4 mm Mobile phase—isocratic Isooctane: 1,4 dioxane (97:3) Flow rate—2 mL min^{-1}	MS-APCI (CID) MS/MS-APCI Positive ion mode	DL—α-T—50 pg. (β + γ)-T—1.0 ng δ-T—5.4 mg	*Chromatographia*, 54, 179, 2001[257]

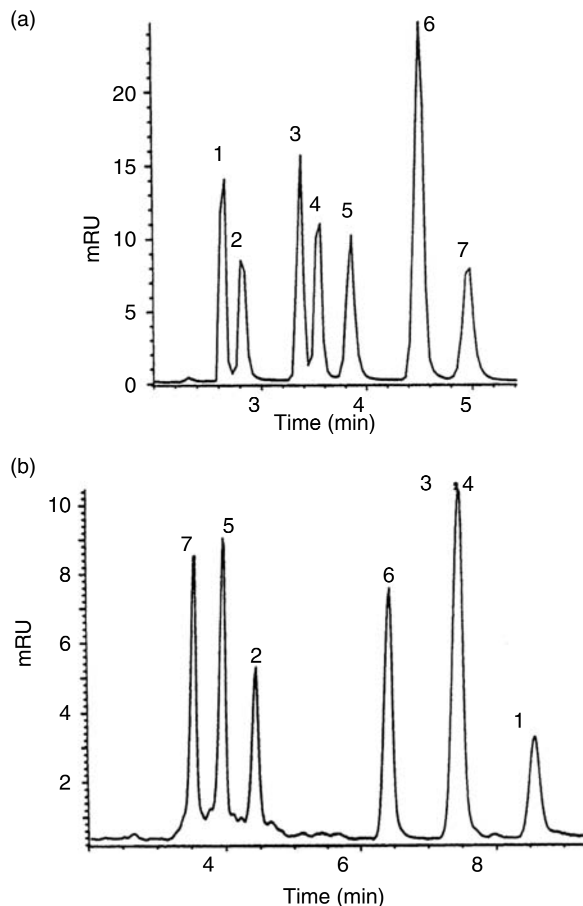

Figure 3.6 Chromatograms of tocopherols and tocotrienols. (a) Normal phase—Zorbax SIL with hexane:isopropanol (99:1). (b) Reversed phase—Zorbax ODS with acetonitrile:methanol:methylene chloride (60:35:5). α-T (1), α-T3 (2), β-T (3), γ-T (4), γ-T3 (5), δ-T (6), δ-T3 (7). (Reproduced from Tan, B. and Brzuskiewicz, L., *Anal. Biochem.*, 180, 368, 1989. With permission.)

compared to mobile phases containing more polar alcohol or polar ether modifiers. Chromatograms given in Figures 3.7 and 3.8 show the differences in a variety of mobile phases to resolve the β- and γ-T dimethyl homologs on diol silica support.

Vitamin E mixtures were highly resolved using an amino-column and hexane: *tert*-butyl ether (90:10) on a 5 mm diol column with hexane: *tert*-butyl methyl ether (95:5). Mobile phases modified with monofunctional ethers were highly recommended by the authors to improve tocopherol and tocotrienol resolution.

Retention factors (*k*), separation factors (α), theoretical plates (*N*), and resolution (*RS*) for normal-phase supports including seven silica columns, two anime columns, and a diol column were reported by Kamal-Eldin et al.[259] Chromatography was compared using a mixture of oat extract, palm oil, and standards to produce a balanced mixture of tocopherols and tocotrienols. Variation exists in the ability of different silica supports to resolve the eight natural vitamin E forms efficiently. Of the seven silica columns, only three effectively resolved the vitamin E mixture. Good separations were also obtained on the amino and diol supports. The results general agreed with Abidi and Mounts's[258] conclusion that weakly polar modifiers provided better selectivity than stronger modifier for resolution of β- and γ-T.

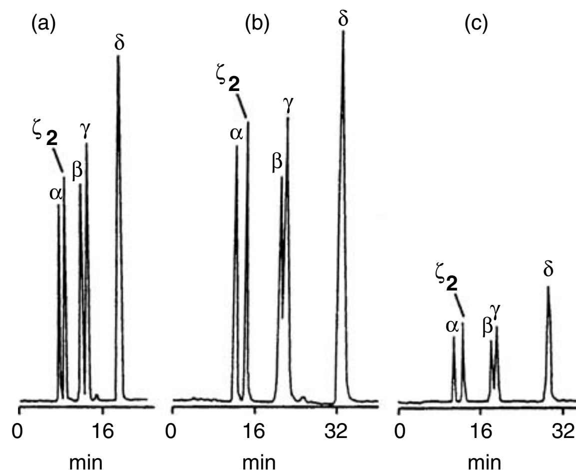

Figure 3.7 Vitamin E chromatography with strongly polar modifiers. (a) amino-Si, hexane:dioxane (90:10); (b) diol-Si (10 μm), hexane:dioxane (95:5); (c) diol-Si (5 μm), cyclohexane:dioxane (97:3). (Reproduced from Abidi, S. L. and Mounts, T. L., *J. Liq. Chromatogr. Rel. Technol.*, 19, 509, 1996. With permission.)

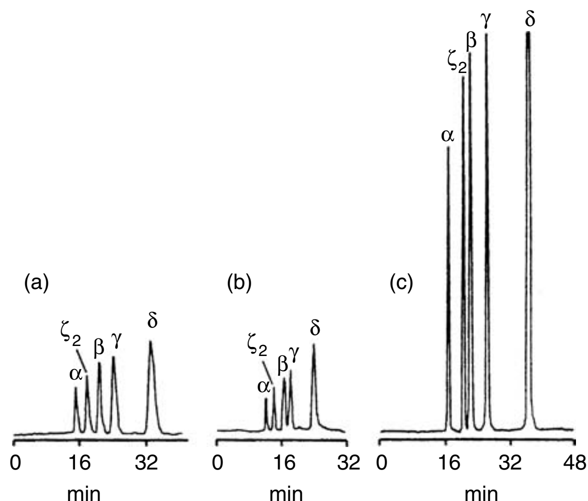

Figure 3.8 Vitamin E chromatography with weakly polar modifiers. (a) amino-Si, cyclohexane:tert-butyl methyl ether (90:10); (b) diol-Si (10 μm), hexane:tert-butyl methyl ether (90:10); (c) diol-Si (5 μm), hexane:diisopropyl ether (90:10). (Reproduced with permission from Abidi, S. L. and Mounts, T. L., *J. Liq. Chromatogr. Rel. Technol.*, 19, 509, 1996. With permission.)

Use of dioxane (4%–5%) in hexane provided good resolution on three of the silica columns examined in the study (Figure 3.9). Normal-phase diol columns have been successfully used to assay tissue and diet levels of tocopherols and tocotrienols.[237]

The ability of narrow-bore (2.1-mm id) silica columns to standard-bore (4.6-mm id) silica columns for chromatography of tocopherols were studied by Ye et al.[260] Narrow-bore columns have some combined characteristics of microbore and standard-bore columns but without the specialized hardware requirements needed for microbore chromatography. Narrow-bore chromatography can be easily implemented on conventional LCs with pumps capable of handling flow rates lower than 1.0 mL min^{-1}. Advantages of microbore columns

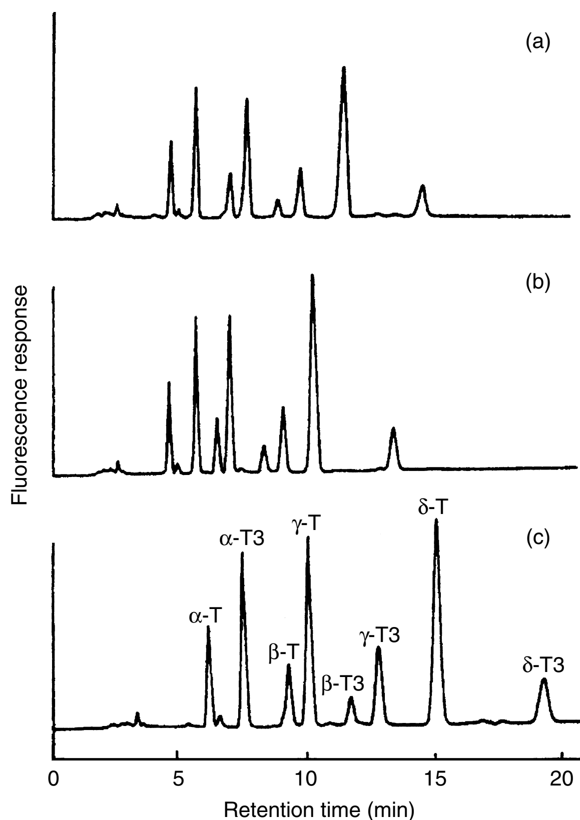

Figure 3.9 Separation of a balanced mixture of tocopherols on three silica columns. (a) Alltima SI, 5 μm, 25 cm × 4.6 mm (Alltech) using hex:dioxane (96:4), 2 mL min^{-1}; (b) Intersil SI, 5 μm, 25 cm × 4.6 mm (Chromapack) using hex:dioxane (95:5), 2 mL min^{-1}; (c) Genesis silica, 4 μm, 25 cm × 4.6 mm (Jones) using hex:dioxane (96:4), 1.5 mL min^{-1}. α-T, α-tocopherol; α-T3, α-tocotrienol. (Reproduced from Kamal-Eldin, A., Görgen, S., Pettersson, J., and Lampi, A. M., *J. Chromatogr. A*, 881, 217, 2000. With permission.)

over standard-bore that carry over to narrow-bore columns include less solvent consumption, reduction in stationary phase amount, and higher mass sensitivity.[261] Conditions used by Ye et al.[260] included mobile phase of 0.8% isopropanol in hexane pumped in 1.0 mL min^{-1} for standard-bore chromatography. Column temperature was controlled at 29°C ± 1°C.

A comparison of column performance statistics is provided in Table 3.10. Notable differences between the two columns include higher theoretical plates, lower back pressure, and better resolution for the standard-bore as compared to the narrow-bore column. Narrow-bore gave higher sensitivity and lower solvent consumption than standard-bore. DL and QL values for the tocopherols are given in Table 3.11. Peaks elute from the narrow-bore column in much smaller volume with less dispersion; therefore, detector signals are of higher intensity because of higher concentration in the detector flow cell. The DL values for the vitamin E homologs were four to seven times lower for the narrow-bore column and up to eight times lower for the QL (Table 3.11). Narrow-bore chromatography results in significant solvent savings with the loss of some resolution. Figure 3.10 shows resolution of the tocopherols and the differences in detector response of eluting peaks.

In the past, reversed-phase LC on C_{18} supports has had limited use for assay of vitamin E in foods owing to the inability to completely resolve the tocopherols and tocotrienols. The C_{30} polymeric support, originally developed to improve carotenoid resolution,[261] has greatly increased the resolution power of reversed-phase chromatography for the

Table 3.10 Analytical Figures of Merit for Chromatography of α-, β-, γ-, and δ-T on Narrow-Bore and Standard-Bore Columns[a,b]

Analytes	r^2	k'	N	T	S	α	R_s
α-T	0.9996	0.78	6036	1.0	1.0		
	(0.9999)	(1.0)	(8.236)	(0.9)	(0.5)	2.0	6.71
β-T	0.9998	1.57	5182	1.1	0.9	(2.0)	(10.3)
	(0.9999)	(2.0)	(11,274)	(1.1)	(0.5)	1.1	1.4
γ-T	0.9998	1.77	5560	1.1	0.9	(1.1)	(2.2)
	(0.9999)	(2.2)	(12,777)	(0.9)	(0.5)	1.8	7.72
δ-T	0.9998	3.19	5905	1.1	1.0	(1.8)	(11.5)
	(0.9999)	(4.0)	(10,630)	(1.0)	(0.5)		

[a] 10-μL injection volume and 0.8% IPA in hexane as mobile phase.

[b] Values in parentheses are values from standard-bore. r^2, Linearity, range 0.33–16.37, 0.15–7.72, 0.31–15.52, 0.34–17.19 ng injection^{-1} (n = 5) for α-, β-, γ-, and δ-T in narrow-bore column, respectively; 0.65–32.74, 0.31–15.43, 0.62–31.03, 0.69–34.38 ng injection^{-1} (n = 5) for α-, β-, γ-, and δ-T in standard-bore column, respectively. α-T, α-tocopherol. k', retention factor; N, theoretical plates; T, tailing factor; S, system suitability, % RSD of 5 replicate injections at 3.23, 1.54, 3.10, and 3.44 ng injection^{-1} in narrow-bore column for α-, β-, γ-, and δ-T, respectively; at 6.55, 3.09, 6.21, and 6.88 ng injection^{-1} in standard-bore column for α-, β-, γ-, and δ-T, respectively. α, separation factor; R_s, resolution.

Source: Ye, L., Landen, W. O., Jr., and Eitenmiler, R. R., Comparison of the column performance of narrow-bore and standard-bore column for the chromatographic determination of α-, β-, γ- and δ-tocopherol, *J. Chromatogr. Sci.*, 39, 1, 2001.

Table 3.11 Limit of Detection (ng), Limit of Quantitation (ng) for the Chromatography of α-, β-, γ-, and δ-Tocopherol on Narrow-Bore and Standard-Bore Columns

	Narrow-bore		Standard-bore			
Analytes	DL	QL	DL	QL	DL	QL
α-T	0.032	0.094	0.021	0.051	0.119	0.307
β-T	0.016	0.046	0.006	0.014	0.029	0.072
γ-T	0.025	0.075	0.012	0.028	0.049	0.119
δ-T	0.039	0.113	0.011	0.024	0.075	0.200
Column	LiChrosorb Si60, 25 cm × 2.1 mm 5 μm		LiChrosorb Si60, 25 cm × 2.1 mm 5 μm		LiChrosorb Si60, 25 cm × 4.6 mm 5 μm	
Flow rate	0.32 mL min^{-1}		0.32 mL min^{-1}		1.0 mL min^{-1}	
MP	0.6% IPA in hexane		0.8% IPA in hexane		0.8% IPA in hexane	

DL: Limit of detection; QL: limit of quantitation; α-T: α-tocopherol; MP: mobile phase; IPA: isopropyl alcohol.

Source: Ye, L., Landen, W. O., Jr., and Eitenmiler, R. R., Comparison of the column performance of narrow-bore and standard-bore column for the chromatographic determination of α-, β-, γ- and δ-tocopherol, *J. Chromatogr. Sci.*, 39, 1, 2001.

tocopherols and tocotrienols.[193,224,248,263–267] Rimmer et al.[267] recently evaluated monomeric and polymeric C_{18}, C_{27}, C_{30}, and immobilized poly(ethylene-co-acrylic acid) stationary phases on the basis of methylene selectivity, silanol activity, metal activity, pore size, and shape selectivity for the supports ability to resolve tocopherol and carotenoid isomers. The work showed that monomeric and polymeric C_{30} supports resolved the tocopherols while polymeric C_{30} and polyethyl supports were better for the resolution of the carotenoids. Figure 3.11 shows resolution of α-, β-, γ-, and δ-T on monomeric and polymeric C_{30} columns.[267]

3.3.3.2.2.2 Detection. Detection of tocopherols and tocotrienols after LC resolution relies on UV, fluorescence, electrochemical (EC), evaporative light scattering (ELSD), and

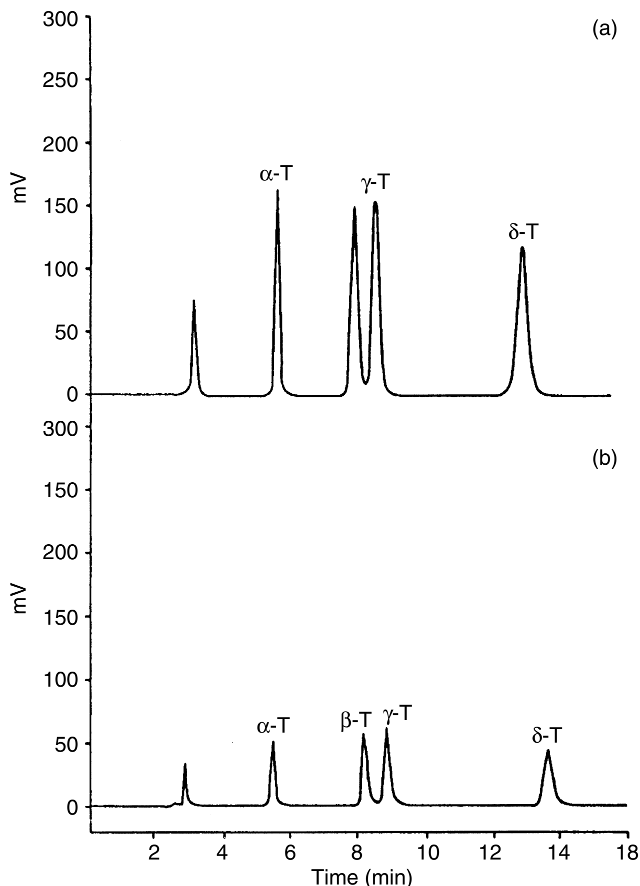

Figure 3.10 Chromatography of tocopherols on (a) 250 × 2.1 mm id LiChrosorb Si60, flow rate 0.32 mL min^{-1}, and (b) 250 × 4.6 mm id LiChrosorb Si60, flow rate 1.0 mL min^{-1}. Mobile phase was 0.8% isopropanol in hexane; injection volume was 10 mL. α-T, α-tocopherol. (Reproduced from Ye, L., Landen, W. O., Jr., and Eitenmiler, R. R., *J. Chromatogr. Sci.*, 39, 1, 2001. With permission.)

mass spectrometry. By far, the most commonly used detector for vitamin E analysis is fluorescence, which is considerably more sensitive and selective than UV but less sensitive than EC. Fluorescence intensity of the eluted vitamin E peaks depends upon many factors; thus, comparison of fluorescence response to UV or EC measures of detector sensitivity is somewhat dependent on equipment and the chemical environment of the mobile phase passing through the detector. A review of detection methods for vitamin E reported that one study showed that sensitivity of fluorescence was 2.5– to 3.3-fold greater than absorbance at 292 nm.[101] This sensitivity differential is probably too low if comparable measurements were performed with new-generation fluorescence detectors with improved engineering and higher-intensity lamps for excitation. Hoehler et al.[238] reported 150- to 340-fold increases in sensitivity of fluorescence compared to UV at 280 nm for the tocopherols. Sensitivity of fluorescence depends a great deal upon the composition of the mobile phase. For example, α-T and retinol have a five- to six fold decrease in fluorescence intensity when the mobile phase is changed from hexane to acetonitrile:water (1:1).[104] Many components of reversed-phase mobile phase mixtures lead to decreased fluorescence of vitamin E components compared to fluorescence intensity possible in normal-phase systems.[268] Polarity of the solvent greatly influences fluorescence intensity of many organic compounds.[269] Caution has to be used to

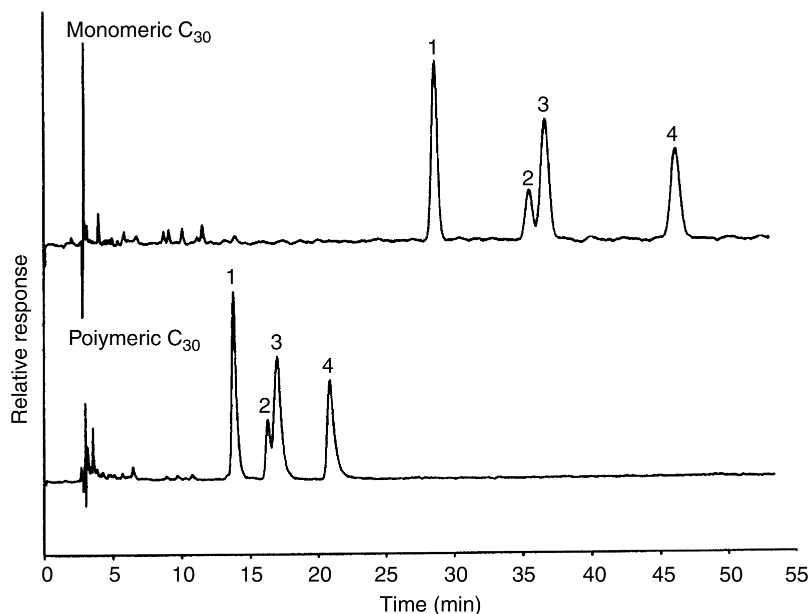

Figure 3.11 Resolution of tocopherols on C_{30} supports. Peak 1—δ-T, Peak 2—γ-T, Peak 3—β-T, Peak 4—α-T. (Reproduced from Rimmer, C. A., Sander, L. C., and Wise, S. A., Selectivity of long chain stationary phases in reversed phase liquid chromatography, *Anal. Bioanal. Chem.*, 382, 698, 2005. With permission.)

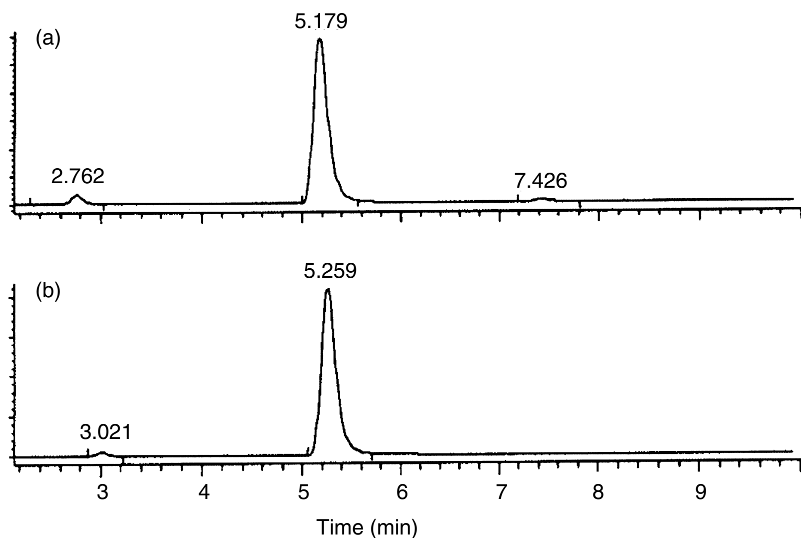

Figure 3.12 LC-MS chromatograms of Nestlé reference sample (milk powder) spiked with (a) α-tocopherol and (b) d_6-tocopherol obtained in SIM mode. (Reproduced from Kalman, A., Mujahid, C., Mottier, P., and Heudi, O., *Rapid Commun. Mass Spectrom.*, 17, 723, 2003. With permission.)

prevent quenching of fluorescence (reduced fluorescence intensity). Factors producing quenching include dissociation of the molecule by the light energy necessary for excitation, absorbance of emitted light by other molecular species or by the analyte itself, and dissipation of energy from collisions of molecules.

Selectivity of fluorescence detection results from the fact that two wavelengths are used in the measurement compared to one for ultraviolet/visible (UV/Vis) detection. Structural features necessary for a molecule to fluoresce are limited in nature, whereas, UV/Vis absorbance is common to most organics. The chromanol ring structure of the tocopherols and tocotrienols with fluorescence properties of maximal emission at 345 nm with excitation at 210 or 290 nm provides a highly selective detection system readily noted from clean fluorescence chromatograms from complex matrices, produced with minimal sample cleanup. Most lipids do not fluoresce,[104] therefore, lipids can be injected directly onto silica columns, normally without interference problems.

Older literature states that tocopheryl esters such as α-tocopheryl acetate do not fluoresce; thereby, limiting the detector of such compounds to UV. However, with the availability of high-intensity excitation sources and new detector design, fluorescence of α-tocopheryl acetate is readily measurable. Reports by Woollard and Blott,[201] Woollard et al.,[202] and Baltz et al.[226] were the first to document the ability to measure α-tocopherol acetate by fluorescence in the assay of infant formula. Since these reports, α-tocopheryl acetate in fortified foods is routinely determined by fluorescence. In this respect, fluorescence has improved assay of vitamin E levels in fortified foods since direct solvent extraction often can be employed in place of saponification, allowing biological activity to be more accurately assessed.

Electrochemical detection in combination with LC resolution is an effective analytical approach. A 1985 review indicates EC is at least 20 times more sensitive than fluorescence for the detection of tocopherols.[151] However, EC is limited to reversed-phase chromatography because of the need for electrolyte in the mobile phase for electrical conductance. Electrochemical detection has been used to assay tocopherols in foods and biologicals. Ueda and Igarashi[153,154] effectively applied EC to analyses of high fat samples. The increased sensitivity of EC detection allowed use of reduced sample amounts; thereby, limiting the effect of sodium salts in the extraction to decrease analyte recovery.

Evaporative light scattering can be used as a detection mode for vitamin E. However, fluorescence is at least ten times more sensitive than ELSD.[185,270] Few studies using ELSD have been completed because of operational cost, instrument complexity, and lower sensitivity.

Nonpolar compounds such as the tocopherols and tocotrienols do not ionize easily.[247,251,271,272] Rentel et al.[271] increased sensitivity of MS detection for vitamin E by addition of silver ions to the LC eluent to form Ag^+-tocopherol adducts that facilitated ionization. This technique was called coordination ion spray (CIS), which allows ESI in the positive ion mode. The tocopherols are detected as the $[M + Ag^+]$ adducts. Strohschein et al.[272] expanded CIS to structural identification of tocotrienol in a palm oil vitamin E concentrate. They identified α-, β-, γ-, and δ-T3, α-tocoenol, and α-T in the concentrate. Al-Talla and Tolley[251] applied CIS-MS to the analysis of α-T in human serum by a fast and sensitive method using direct infusion of the serum extract into the mass spectrometer.

Ionization difficulties noted for the vitamin E compounds has produced continued studies leading to improvements of MS applications in addition to the application of CIS. Traber's research group at Oregon State University initially quantified vitamin E in biological samples by LC-MS/MS using APCI in the positive ion-MRM mode.[246] They noted that α-T generated ions at *m/z* 430 and *m/z* 429 instead of the expected $[M + H^+]$ ion at *m/z* 431. Formation of the *m/z* 430 molecular ion in the APCI source indicated a one-electron oxidation of α-T; whereas, formation of the species at *m/z* 429 $[M - H]^+$ showed protonation of α-T to the $[M + H]^+$ ion, followed by dehydrogenation. This work was followed by improved analyses using LC-MS/MS-APCI in the negative ionization mode[249] and LC-MS-ESI in the negative ionization mode.[250] For the LC-MS/MS-APCI method, sensitivity was increased

10–20 times compared to the earlier assay using the positive ion mode. Earlier studies by Hall et al.[245] showed the advantages of negative ion mode ESI for vitamin E analysis using MS.

3.3.3.2.3 Internal standards. For some matrices, internal standards can simplify vitamin E analysis. For serum and plasma, δ-tocopherol is normally not detectable in the sample and can be used as an excellent internal standard for either UV or fluorescence detection. α-Tocopheryl acetate, with UV detection or a sensitive fluorescence detector, is suitable. Several studies used tocol as an internal standard; however, analytical problems are associated with its use. Ueda and Igarashi[155] determined tocol to be an unsuitable internal standard for saponification procedures owing to low recoveries in relation to the analytes. These investigators introduced 2,2,5,7, 8-pentamethyl-6-chromanol (PMC) as an internal standard for vitamin E methods requiring saponification.[154] 5,7-Dimethyltocol has been used in some studies.[237,258] Tocotrienols, because of their absence from most foods and biological samples, could be effectively applied as internal standards. Under all circumstances, internal standards must be added before saponification or solvent extraction.

In recent studies conducted using LC-MS or LC-MS/MS, deuterated α-T (d_3-*RRR*, d_6-all-*rac*),[140,246,249] deuterated α-tocopheryl acetate (d_6-*RRR*-α-TAC),[206,246,249] and deuterated α-tocopherol quinone (d_3)[140] are excellent internal standards. α-Tocopheryl succinate was a suitable internal standard for CIS-MS.[251] Deuterated internal standards provide compounds closely related to the analytes' chemical and chromatographic characteristics; but, mass differences from the nondeuterated analytes allow mass spectrometric detection and can greatly improve accuracy of the assay.[206]

3.4 Method protocols

Analysis of Vitamin E in Food Products Using Liquid Chromatography

Peanut Sci., 20, 21, 1993;[216] *Am. Oil Chem. Soc.*, 70, 633, 1993;[217] *J. Food Compos. Anal.*, 19, 196, 2006.[44]

Principle

Fruit, Vegetables, Meat: Products were saponified with KOH for 30 min with reflux at 70°C and the unsaponifiable materials extracted with hexane (0.1% BHT). The combined organic phases were diluted to volume and 20 mL was injected onto a normal-phase LC column connected to a fluorescence detector (Ex λ = 290, Em λ = 330).

Margarine and Vegetable Oil Spreads: Products were dissolved in hexane and $MgSO_4$ was added to remove water. The extracts were filtered and diluted to volume in hexane (0.1% BHT) for normal-phase LC analysis with fluorescence detection (Ex λ = 290, Em λ = 330).

Reagents and Solvents

- 0.1% BHT in hexane for extraction
- 6% pyrogallol in ethanol for antioxidant
- Ethanol
- Potassium hydroxide
- α-Tocopherol standard—E1% 1 cm = 71 at 294 nm
- γ-Tocopherol standard—E1% 1 cm = 92.8 at 298 nm
- β-Tocopherol standard—E1% 1 cm = 86.4 at 297 nm
- δ-Tocopherol standard—E1% 1 cm = 91.2 at 298 nm
- $MgSO_4$ for margarine and vegetable oil spreads
- Sodium chloride
- Compressed nitrogen

Apparatus

- Saponification flask equipped with reflux condenser
- Bell jar filtration apparatus for margarine and vegetable oil spreads
- Liquid chromatograph
- Fluorescence detector (Ex λ = 290, Em λ = 330)
- Millipore filtration apparatus

Procedure

Vegetable, Fruit, and Meat Products

- Add 10 mL 6% (w/v) pyrogallol to sample weight, mix, and flush with N_2.
- Heat at 70°C for 10 min with sonication.
- Add 2 mL 60% KOH solution, mix, and flush with N_2.
- Digest for 30 min at 70°C under reflux.
- Sonicate 5 min.
- Cool to room temperature, add sodium chloride and water.
- Extract with hexane (0.1% BHT) three times.
- Add 0.5 g $MgSO_4$, mix.
- Filter through Millipore filtration apparatus (0.45 mm).
- Dilute to volume with hexane.
- Inject 20 mL.

Margarine and Vegetable Oil Spreads

- Add 40 mL hexane (0.1% BHT) to 10 g sample and mix.
- Add 3 g $MgSO_4$, mix, let stand, 2 h.
- Filter and dilute combined filtrate to volume with hexane (0.1% BHT).
- Inject 20 mL.

Chromatography

Column	25 cm × 4.6 mm
Stationary phase	LiChrosorb Si60, 5 μm
Mobile phase	0.9% isopropanol in hexane
Column temperature	Ambient
Flow rate	1 mL min^{-1}
Injection	20 mL
Detector	Fluorescence, Ex λ = 290, Em λ = 330
Calculation	External standard, peak area, linear regression

Special Comments

- Determine tocopherol recovery for each food product.
- Sample weight may vary between 1 and 10 g depending on oil content and vitamin level, with lipid content of the sample at less than 0.2 g.
- Before hexane extraction, add water if necessary to reduce alcohol below 30%, v/v.
- For some food products such as raw shrimp, raw chicken, raw fish, it is necessary to blend with sea-sand (hexane-washed) to prevent clumping and aid in KOH digestion.
- For margarine and vegetable oil spreads, the amount of $MgSO_4$ added varies, depending on water content, 1 g for each gram water plus 1 g excess.
- For vegetable oil spreads containing $ 50% fat, blend product at room temperature to prevent separation of water and fat before weighing sample.

Vitamin E Content of Margarine and Reduced Fat Products Using a Simplified Extraction Procedure and HPLC Determination

J. Liq. Chromatogr. Rel. Technol., 21, 1227, 1998.[172]

Liquid Chromatographic Method
Principle

Tocopherol is extracted in hexane with anhydrous magnesium sulfate added to remove water. Vitamin E is quantitated by normal-phase LC with fluorescent detection (Ex λ = 290, Em λ = 330).

Reagents and Solvents

- 0.1% BHT in hexane for extraction
- Isopropanol
- $MgSO_4$
- α-, γ-, and δ-tocopherol standards
- Polyoxyethylene sorbitan monooleate (Tween 80)

Apparatus

- Bell jar filtration apparatus
- Liquid chromatograph
- Sonicator
- Fluorescence detector

Procedure

Sample Preparation and Extraction

- Weigh 5.0 g margarine or spread
- Add 40 mL hexane (0.1% BHT)
- Sonicate
- Rinse flask with 10 mL hexane (0.1% BHT)
- Add 3 drops of Tween 80
- Add 3 g $MgSO_4$
- Let stand, 2 h
- Filter
- Quantitatively transfer filtrate to 100 mL volumetric flask
- Dilute to volume with hexane (0.1% BHT)
- Transfer 1.0–50 mL volumetric flask
- Dilute to volume with hexane (0.1% BHT)
- Inject extract

Chromatography

Column	25 cm × 4.6 mm
Stationary phase	LiChrosorb Si60, 5 µm
Mobile phase	0.9% Isopropanol in hexane
Column temperature	Ambient
Flow rate	0.9 mL min^{-1}
Injection	20 mL
Detector	Fluorescence, Ex λ = 290, Em λ = 330
Calculation	External standard, peak area, linear regression

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chapter four

Vitamin K

4.1 Review

The existence and basic function of vitamin K was determined in the 1930s through the efforts of several research groups. Original observations by Dam in Denmark showed that chicks developed blood with poor clotting properties along with hemorrhages when fed diets extracted with ether. Addition of the ether extract back into the diet improved the condition of the chicks.[1] Dam named the fat-soluble, antihemorrhagic factor "vitamin K" from the word "koagulation." Vitamin K was isolated by Dam from alfalfa and identified as 2-methyl-3-phytyl-1,4-naphthoquinone. The designation K_1 was given along with the generic name "phylloquinone." Vitamin K_2, the menaquinone-n (MK-n) form, was crystallized from bacterial fermented fishmeal by Doisy.[2] The menaquinones are 2-methyl-1,4-naphthoquinones substituted at position 3 with an unsaturated isoprenyl side chain of varying numbers of isoprenoid units. Doisy synthesized vitamin K_1 in 1939. In 1943, Dam and Doisy received Nobel Prizes for their work on the discovery of vitamin K (Dam) and its synthesis (Doisy).[3]

In the human, vitamin K deficiency commonly occurs in the newborn and is named hemorrhagic disease. The deficiency is not due to poor nutrition of the mother, but to poor placental transfer, absence of bacterial synthesis in the newborn's gut, low plasma concentrations of plasma clotting factors, and low vitamin K concentrations in human milk.[4] The deficiency is characterized by low plasma prothrombin concentrations resulting from the lack of biosynthesis of prothrombin by the immature liver and the lack of vitamin K. Hemorrhagic disease produces bleeding in the skin, subcutaneous tissue, gastrointestinal (GI) tract, umbilical cord, and intracranium. Central nervous system damage occurs and the deficiency is often fatal.[5] Hemorrhagic disease is prevented by intramuscular injection or oral administration[4,6,7] of vitamin K_1 within 6 h of birth. Breast-fed infants are more likely to develop hemorrhagic disease than formula-fed infants because human milk is low in vitamin K content. Infant formula is fortified at a minimum level of 4 mg per serving specified by the Infant Formula Act of 1980.[8]

Deficiency of vitamin K in the adult is rare. When it does occur, the deficiency is defined as a "vitamin K-responsive hypoprothrombinemia,"[9] resulting from fat malabsorption syndromes, liver disease, and antibiotic therapy that inhibits microbial vitamin K_2 synthesis in the gut. Anticoagulant treatment with coumarin and related compounds produces a functional or secondary vitamin K deficiency through disruption of the vitamin K cycle and inhibition of the synthesis of vitamin K-dependent blood clotting proteins in the liver. Defective coagulation of the blood measured by a one-stage prothrombin time (PT) is still used to assess vitamin K status; however, the test is not a sensitive indicator of vitamin K status.[8] Coagulation assays are not useful to assess subclinical deficiencies.[10] Direct measurement of gamma-carboxylation status of gamma-carboxyglutamate (Gla) proteins that are

vitamin K-dependent provides a biochemical marker to assess vitamin K status.[9,10] Analytes include undercarboxylated prothrombin proteins (hepatic vitamin K status) and osteocalcin (bone vitamin K status).[8,9] Serum and urinary levels of vitamin K do not reflect tissue status.[10]

Information on the vitamin K content of the food supply has been greatly improved through research conducted by Sadowski and colleagues[11–23] and Booth and colleagues.[24–36] This work not only provided methodology necessary to accurately measure vitamin K_1 in foods, but also presents usable food composition tables[13] and information on vitamin K_1 intake.[15–17,19,20,22,24,25,31,36] Table 4.1 summarizes vitamin K_1 intake in women's diets collected from the 1990 Food and Drug Administration Total Diet Study.[17] Major top five contributors of vitamin K were spinach, collards, broccoli, iceberg lettuce, and coleslaw with dressing. Addition of fats and oils to mixed dishes and desserts was considered an important source of vitamin K_1 in the American diet.

Leafy green vegetables, certain legumes, and vegetable oils are considered good sources of vitamin K_1.[37] Milk and dairy products, meats, eggs, cereals, fruits, and vegetables are considered low but consistent, and significant sources of vitamin K to the American diet.[9] Table 4.2 gives food composition information for vitamin K in μg 100 g^{-1} derived from the United States Department of Agriculture National Nutrient Database for Standard Reference, Release 19.[38] The relatively high amount of vitamin K in leafy green vegetables compared to other common food sources is readily noted from this data. Much of the composition data on vitamin K is derived from the work of the Sadowski and Booth research groups. Recent research showed that breads, rice, and pastas are poor sources of vitamin K unless formulated with vegetable oils.[35] Addition of fats and oils to mixed dishes is a significant source of vitamin K_1.[24] Since human milk is low in vitamin K, infant formula in the

Table 4.1 Major Contributors of Vitamin K_1 in Women's Diets: Data from the 1990 Total Diet Study

	Vitamin K_1	
Rank/food description	% Total	% Cumulative
1. Spinach, fresh/frozen, boiled	13.1	13.1
2. Collards, fresh/frozen, boiled	11.9	25.0
3. Broccoli, fresh/frozen, boiled	9.8	34.8
4. Iceberg lettuce, raw	6.9	41.7
5. Coleslaw with dressing, homemade	5.6	47.3
6. French salad dressing, regular	3.2	50.5
7. Cabbage, fresh, boiled	3.0	53.5
8. Green beans, fresh/frozen, boiled	2.3	55.8
9. Brussels sprouts, fresh/frozen, boiled	2.3	58.1
10. Green peas, fresh/frozen, boiled	2.1	60.2
11. Margarine, stick, regular	1.9	62.1
12. Tuna, canned in oil, drained	1.6	63.7
13. Beef chow mein, carryout	1.5	65.2
14. Asparagus, fresh/frozen, boiled	1.5	66.7
15. Mayonnaise, regular, bottled	1.4	68.1
16. Carrots, fresh, boiled	1.2	69.3
17. Eggs, scrambled	1.1	70.4
18. Apple pie, fresh/frozen, commercial	1.1	71.5
19. Taco/tostada, carryout	1.1	72.6
20. Mashed potatoes from flakes	1.0	73.6

Source: Reproduced from Booth, S. L., Pennington, J. A. T., and Sadowski, J. A., *J. Am. Diet. Assoc.*, 96, 149, 1996. With permission.

Table 4.2 Vitamin K Content of Various Foods[a]

Description	NDB No	µg 100 g^{-1}	Description	NDB No	µg 100 g^{-1}
Parsley, raw	11297	1640.0	Cabbage, savoy, raw	11114	68.9
Spices, parsley, dried	02029	1361.5	Cabbage, raw	11109	60.0
Kale, frozen, cooked, boiled, drained, without salt	11236	895.8	Plums, dried (prunes), uncooked	09291	59.5
Collards, frozen, chopped, cooked, boiled, drained, without salt	11164	623.2	Fast foods, coleslaw	21127	57.0
Turnip greens, frozen, cooked, boiled, drained, without salt	11575	518.9	Asparagus, cooked, boiled, drained	11012	50.7
Beet greens, cooked, boiled, drained, without salt	11087	484.0	Fish, tuna, light, canned in oil, drained solids	15119	44.0
Spinach, raw	11457	483.0	Asparagus, canned, drained solids	11015	41.3
Spinach, canned, drained solids	11461	461.6	Kiwi fruit (Chinese gooseberries), fresh, raw	09148	40.3
Turnip greens, cooked, boiled, drained, without salt	11569	367.6	Okra, cooked, boiled, drained, without salt	11279	40.0
Mustard greens, cooked, boiled, drained, without salt	11271	299.5	Cabbage, red, raw	11112	38.1
Endive, raw	11213	231.0	Celery, cooked, boiled, drained, without salt	11144	37.8
Onions, spring or scallions (includes tops and bulb), raw	11291	207.3	Rhubarb, frozen, cooked, with sugar	09310	29.6
Onions, spring or scallions (includes tops and bulbs), raw	11291	207.0	Celery, raw	11143	29.3
Brussel sprouts, frozen, cooked, boiled, drained, without salt	11101	193.5	Cowpeas (Blackeyes), immature seeds, cooked, boiled, drained, without salt	11192	26.6
Lettuce, green leaf, raw	11253	174.0	Lettuce, iceberg (includes crisphead types), raw	11252	24.1
Lettuce, green leaf, raw	11253	173.6	Blueberries, raw	09050	19.3
Broccoli, cooked, boiled, drained, without salt	11091	141.1	Blueberries, frozen, sweetened	09055	17.7
Vegetable oil, canola	04582	122.1	Cucumber, with peel, raw	11205	16.4
Salad dressing, French dressing, commercial, regular	04120	121.2	Cauliflower, raw	11135	16.0
Lettuce, cos or romaine, raw	11251	102.5	Pumpkin, canned, without salt	11424	16.0
Broccoli, raw	11090	101.6	Carrot juice, canned	11655	15.5
Salad dressing, home recipe, vinegar and oil	04135	98.7	Beans, snap, yellow, frozen, cooked, boiled, drained, without salt	11732	12.7
Asparagus, frozen, cooked, boiled, drained, without salt	11019	80.0	Beans, snap, green, frozen, cooked, boiled, drained, without salt	11061	12.7

[a] Data from USDA National Nutrient Database for Standard Reference, Release 19[38].

Table 4.3 Dietary Reference Intakes for Vitamin K

Life stage	DRI (μg d^{-1})
Infants (months)	
0–6	2.0
7–12	2.5
Children (years)	
1–3	30
4–8	55
Males (years)	
9–13	60
14–18	75
19–30	120
31–50	120
51–70	120
>70	120
Females (years)	
9–13	60
14–18	75
19–30	90
31–50	90
51–70	90
>70	90
Pregnancy (years)	
≤18	75
19–30	90
31–50	90
Lactation (years)	
≤18	75
19–30	90
31–50	90

Source: Food and Nutrition Board, Institute of Medicine, *Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron Manganese, Molybdenum, Nickel, Silicon, Vandium and Zinc*, National Academy of Sciences Press, Washington, DC, 2002, Chap. 4.

United States is fortified at a minimum level of 4 μg 100 $kcal^{-1}$ as specified by the Infant Formula Act of 1980.[39]

Dietary Reference Intake (DRI) values for vitamin K are given in Table 4.3. The DRI committee considered intake data too limited to estimate an average requirement; therefore, Adequate Intake (AI) recommendations were given for all age and gender groups.[9] The AI levels range from 2.0 μg d^{-1} for infants 0–6 months of age to 120 μg d^{-1} for males 19 years and older. Tolerable upper intake levels (ULs) were not established, since no adverse effects have been reported at high intake levels. Intake data presented in the DRI report[9] from the Third National Health and Nutrition Examination Survey (NHANES III, 1988–1994)[40] estimated intake at 79–88 μg d^{-1} for women and 89–117 μg d^{-1} for men. Studies by Booth et al.[17] estimated that 25- to 30-year-old men and women consumed 66 and 59 μg d^{-1} of vitamin K_1 (phylloquinone), respectively. Food intake surveys conducted by Booth et al.[41,42] over 5- and 14-day intake periods showed that intakes of vitamin K_1 greatly varied from day to day. The average

intake was 70–80 μg d^{-1} for adults. Most intake estimates for the U.S. population are below recommended AI levels when intake from supplements is not considered.

Vitamin K is a cofactor for vitamin K-dependent (VKD) carboxylase required for the posttranslational conversion of glutamic acid to gamma-carboxy-glutamyl residues (Gla).[4,43] Numerous Gla-containing or VKD proteins are known. These include the blood coagulation proteins consisting of prothrombin (factor II, factors VII, IX, and X, and proteins C, S, and Z).[4] Three VKD proteins including osteocalcin, matrix Gla protein, and protein S[4,43] are bone matrix components. Various other Gla proteins have been identified, but the functions for such VKD proteins are not clearly understood.[4] Roles for which scientific evidence exists include inhibition of calcification, inhibition of cell apoptosis and proliferation, signal transduction, phagocytosis, and transcriptional regulation of bone-specific genes.[44–47]

4.2 Properties

4.2.1 Chemistry

4.2.1.1 General properties

Chemically, vitamin K refers to 2-methyl-1,4-naphthoquinone and all derivatives providing the antihemorrhagic activity of vitamin K_1, phylloquinone.[48] Figure 4.1 shows the structure of 2-methyl-1,4-naphthoquinone, which is the synthetic and simplest form of vitamin K, known as menadione or vitamin K_3 (MK-O). Menadione (Figure 4.1) is the correct name for the compound, which was formerly called "menaquinone." This synthetic form of vitamin K is still designated vitamin K_3. Phylloquinones or vitamin K_1 compounds (Figure 4.1) are produced by plants. Alkylation at position 3 of the 2-methyl-1,4-naphthoquinone ring with five-carbon isoprenoid units produces the vitamin K series found in nature. $K_{1(20)}$, the most common phylloquinone, contains four isoprenoid units—three of which are reduced. The reduced side chain or "phytyl" side chain with one double bond characterizes the phylloquinones. Phylloquinones are, therefore, classified chemically as 2-methyl-3-phytyl-1, 4-naphthoquinones. United States Pharmacopeial Convention (USP) designates vitamin K_1 as phytonadione. USP phytonadione is a mixture of *E* and *Z* isomers containing not more than 21% *Z* isomers. Natural phylloquinone is 2′-*E*,7′*R*, and 11′*R*.[1]

Vitamin K compounds of the menaquinone-n series (MK-n) vitamin K_2 (Figure 4.1) have polyisoprenyl side chains at position 3. Such compounds are synthesized by bacteria and not by plants. Side chains are unsaturated and usually contain 4 to 13 prenyl groups. The MK-n designation indicates the number of prenyl groups in the side chain. One or more of the isoprene units might be hydrogenated,[1] usually at the second isoprenoid unit from the naphthoquinone nucleus.

Physical properties of phylloquinone, MK-4, MK-6, MK-7, and menadione are given in Table 4.4. Vitamin K compounds other than modified menadiones are soluble in lipids, ether, and other nonpolar organic solvents. They are sparingly soluble in polar organic solvents. Vitamin $K_{1(20)}$ is synthesized commercially for use in infant formula, medical foods, and pharmaceuticals. Vitamin $K_{1(20)}$ is used in olestra-containing products according to 21CFR171.867.[50] Menadione and its derivatives are important additives in the feed industry. Menadione is not used for human supplements owing to toxicity. Vitamin K_1 is not used in animal feed owing to its cost, and menadione provides an inexpensive alternative.[51] Menadione addition to feeds is particularly important to poultry rations since chemotherapeutic agents against coccidiosis and parasitic diseases inhibit intestinal synthesis and increase the dietary requirements of the chicken.[51] Various stabilized forms of menadione that are water-soluble are available to the industry. These include menadione sodium bisulfate (MSB), menadione sodium bisulfite complex (MSBC), and menadione dimethylpyrimidinol bisulfite (MPB). Structures are given in Figure 4.1. MSB is the addition product

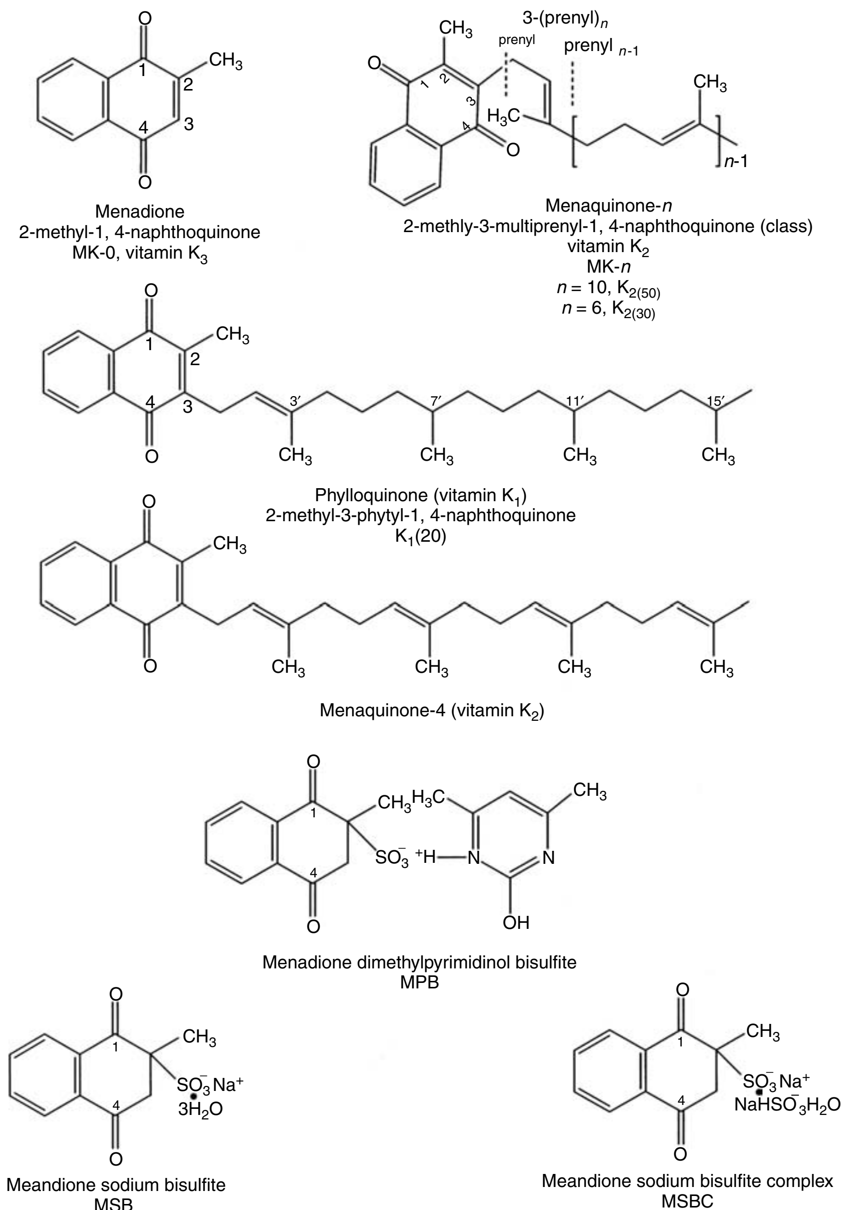

Figure 4.1 Structures of vitamin K and water-soluble menadione forms.

formed between menadione and sodium bisulfite. The product is soluble in water and insoluble in most organic solvents. MSBC is the crystallization product of MSB as a complex in excess sodium bisulfite. MPB is the salt of MSB and dimethylpyrimindinol. The menadione salts are absorbed more efficiently than menadione because of their greater water solubility.[52] The water-soluble menadione forms show better stability to environmental and feed processing conditions compared to free menadione.[52]

4.2.1.2 Spectral properties

Phylloquinones and menaquinone-*n* forms show ultraviolet (UV) spectra characteristic of the naphthoquinone ring. The UV spectrum of phylloquinone shows absorption maxima at 242, 248, 260, 269, and 325 nm. $E^{1\%}_{1\mathrm{cm}}$ values are provided in Table 4.4. Vitamin K compounds

Table 4.4 Physical Properties of Vitamin K

Substance[a]	Molar mass	Formula	Solubility	Melting point (°C)	Crystal form	Spectral properties[b]		
						λ_{max} (nm)	$E^{1\%}_{1\,cm}$	$\varepsilon \times 10^{-3}$
Phylloquinone	450.71	$C_{31}H_{46}O_2$	Sparingly soluble in methanol	—	None	242	396	[17.9]
(Vitamin K_1)			Soluble in ethanol, acetone,		Yellow oil	248	419	[18.9]
CAS No. 84-80-0			benzene, petroleum ether, hex-			260	383	[17.3]
7536			ane, $CHCl_3$, ether			269	387	[17.4]
						325	68	[3.1]
Menaquinone-4	444.70	$C_{31}H_{40}O_2$		35	Yellow crystals	248	439	[19.5]
5876								
Vitamin K_1								
Mk-4								
Menaquinone-6	580.0	$C_{41}H_{56}O_2$		50	Yellow crystals	243	304	[17.6]
5876						248	320	[18.6]
Vitamin K_2						261	290	[16.8]
Mk-6						270	292	[16.9]
						325–328	53	[3.1]
Menaquinone-7	649.0	$C_{46}H_{64}O_2$		54	Micro-yellow	243	278	[18.0]
5876					Crystalline	248	195	[19.1]
Vitamin K_2					plates	261	266	[17.3]
Mk-7						270	467	[30.3]
						325–328	48	[3.1]
Menadione	172.18	$C_{11}H_8O_2$	Insoluble in water	105–107	Bright-yellow			
(Vitamin K_3)			Moderately soluble in $CHCl_3$		crystals			
CAS No. 58-27-5								
5874								

[a] Common or generic name; CAS No: Chemical Abstract Service number; bold print designates the Merck Index Monograph number.
[b] In petroleum ether; values in brackets are calculated from corresponding $E^{1\%}_{1cm}$ values.
Source: Budavari, S., *The Merck Index*, 13th ed., Merck and Company, Whitehouse Station, New Jersey, 2001.

do not fluoresce; however, fluorescence can be induced through reduction of the quinone to hydroquinone. This property has been incorporated into liquid chromatography (LC) methodology to provide a highly specific detection system (Section 4.3.3.2.2.2). The hydroquinone shows maximal fluorescence at Ex $\lambda = 244$, Em $\lambda = 418$.

4.2.2 Stability

Vitamin K_1 is quite stable to oxidation and most food processing and food preparation procedures.[37] It is unstable to light and alkaline conditions. Instability to alkalinity prohibits the use of saponification for sample extraction and led to extensive research to develop sample cleanup procedures to overcome insufficiencies of UV detection for LC methodology (Section 4.3). Reducing agents also destroy the biological activity of vitamin K_1. Isomerization of the *trans-* bond in the 2′-*E*, 7′*R*, and 11′*R* system to 2′-*Z*, 7′*R*, and 11′*R* isomer is problematic because the *cis*-isomer possesses no biological activity.[1] Presence of variable quantities of the *cis*-isomer in commercial vitamin K_1 preparations used for food fortification requires that methodology must accurately assess amounts of *cis*- and *trans*-isomers to estimate biological activity. As discussed in Section 4.3, current Association of Official Analytical Chemists (AOAC) International methods do not meet this requirement.

Few definitive studies exist on vitamin K stability owing to our past inability to accurately quantitate the vitamin. Development of excellent LC procedures has changed this situation. Methods developed in the 1980s and early 1990s have dramatically improved available vitamin K assay methods. In 1992, Ferland and Sadowski[11] reported the effects of heat and light exposure on the stability of vitamin K_1 in vegetable oils. The vitamin was stable during processing and slightly unstable during heating at frying temperatures of 185–190°C for 40 min. The significant findings of the study related to the degree of instability noted on exposure to daylight and fluorescent light. Various conditions were studied and the reader is urged to refer to the complete reference for a complete discussion of the results. However, extensive vitamin K_1 losses approaching 100% were noted in some plant oil after only two days of exposure to fluorescent light. The authors stressed that from an analytical standpoint, it is necessary to work in subdued light when foods are being analyzed. For the consumer, vitamin K_1 content of oils can vary depending upon time and conditions of the marketing channel.

The Sadowski research group reported on the presence of endogenous 2′,3′-dihydro-vitamin K_1 in hydrogenated vegetable oils.[18–20] The physiological importance of the conversion of vitamin K_1 to dihydro-vitamin K_1 is not known, because its biological activity is unknown.[18,19] Owing to the intake of hydrogenated oils, dihydro-vitamin K_1 in the U.S. diet is quite high. Booth et al.[20] reported that intake of dihydro-vitamin K_1 ranged from 12 to 24 mg d^{-1} for all age groups except infants. Vitamin K_1 intake ranges from 24 to 86 mg per day for infants. These authors concluded that if 2′,3′-dihydro-vitamin K_1 does not contribute to vitamin K nutritional needs, hydrogenation of plant oils may be reducing the already low vitamin K intakes.[17,19]

4.3 Methods

4.3.1 General approach

Reviews on vitamin K analysis include Eitenmiller and Landen,[37] Ball,[51] Berruti,[52] and Fauler et al.[53] Berruti's review published in 1985 is informative on older methods for menadione analysis including the titrimetric method with ceric sulfate and colorimetric assays with ethylcyanoacetate and 2,4-dinitro-phenylhydrazine.

Methods based on gas liquid chromatography (GC) are available for phylloquinone, menaquinone-n, and menadione analysis. Reviews by Ball[51] and Fauler et al.[53] give detailed

information on GC methods for vitamin K analysis. Recognized problems of long retention time, high column temperatures, and the potential for on-column degradation have hindered the routine application of GC to vitamin K assay. However, Fauler et al.[53] emphasized that coupling GC with mass spectrometry (GC-MS) provides excellent methodology for analysis of vitamin K in biological samples. Most GC procedures use adaptations of original work by Bechtold and Jahnchen[54] for the separation of vitamin $K_{1(20)}$ and MK-4 on 3% OV-17 with electron capture detection. The low volatility of vitamin K compounds requires high column temperatures. Bechtold and Jahnchen[54] used 302°C. Other published methods use temperature gradients up to 310°C.[55,56] GC allows direct coupling to a mass spectrometer for exact identification and sensitive quantitation. Fauler et al.[55] published a GC-MS method for plasma analysis that combined mass-selective detection with isotope dilution for internal standardization. Degradation of vitamin K_1 was avoided by esterification through a reductive acylation step by the simultaneous addition of zinc dust, heptafluorobutyric anhydride, and heptafluorobutric acid. The detection limit was 1.0 pg and the quantitation limit was 2.0 pg $mL.^{-1}$ Chromatograms and mass spectra from the Fauler et al.[55] study are given in Figures 4.2 and 4.3. Deuterium-labeled vitamin K_1 was synthesized for the study. The procedure quantitated both the 2′-*Z* and natural 2′-*E* isomers in standards at a ratio of 20:80. The 2′-*Z* isomer was not detected in plasma samples.

Recently, Jones et al.[57] modified the Fauler et al.[55] procedure by replacing heptafluorobutyric acid in the derivatization step with heptafluoropropionic acid to form the pentafluoropropionyl ester. Extraction from plasma used enzyme digestion with lipase from *Candida rugosa* and *Pseudomonas* sp. cholesterol esterase, deproteination with ethanol precipitation, extraction of the lipid fraction with hexane, and solid-phase extraction on Sep-Pak-RC^R cartridges following the procedure of Wang et al.[58] Chromatography was on a DB5-MS fused-silica capillary column. Use of methyl-^{13}C and ring D_4 stable isotopes of vitamin K_1 allowed determination of isotope ratios in vitamin K_1 from plasma and eliminated the need for semipreparative LC cleanup used in prior studies to using GC-MS to determine isotope ratios of vitamin K_1 in human plasma.[59,60]

Dramatic improvements have evolved in the application of LC to the determination of vitamin K in biologicals. Earlier procedures were primarily based on UV detection, which were hindered by lack of specificity causing poor chromatographic profiles and the need for complicated and somewhat unwieldy cleanup procedures. Methods developed to overcome problems presented by UV detection incorporated reductive conversion of the

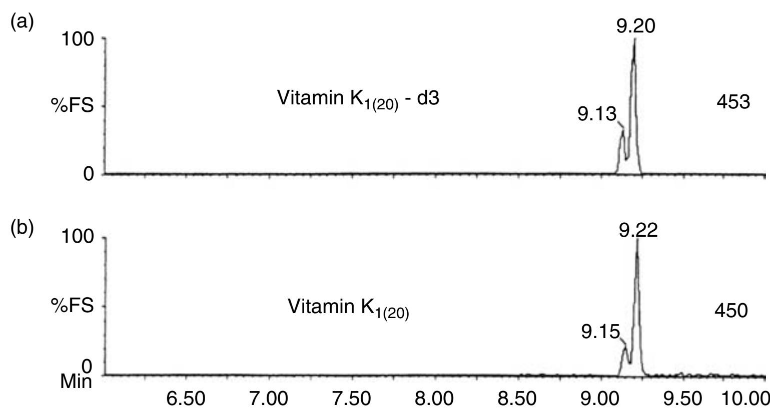

Figure 4.2 SIR mass chromatograms of unlabelled (b) and deuterium-labeled (a) vitamin $K_{1(20)}$ without derivatization. The double peaks correspond to the *cis*- and *trans*-isomers of vitamin $K_{1(20)}$. (Reproduced from Fauler, G., Leis, H., Schalamon, J., Muntean, W., and Gleispach, H., *J. Mass Spectrom.*, 31, 655, 1996. With permission.)

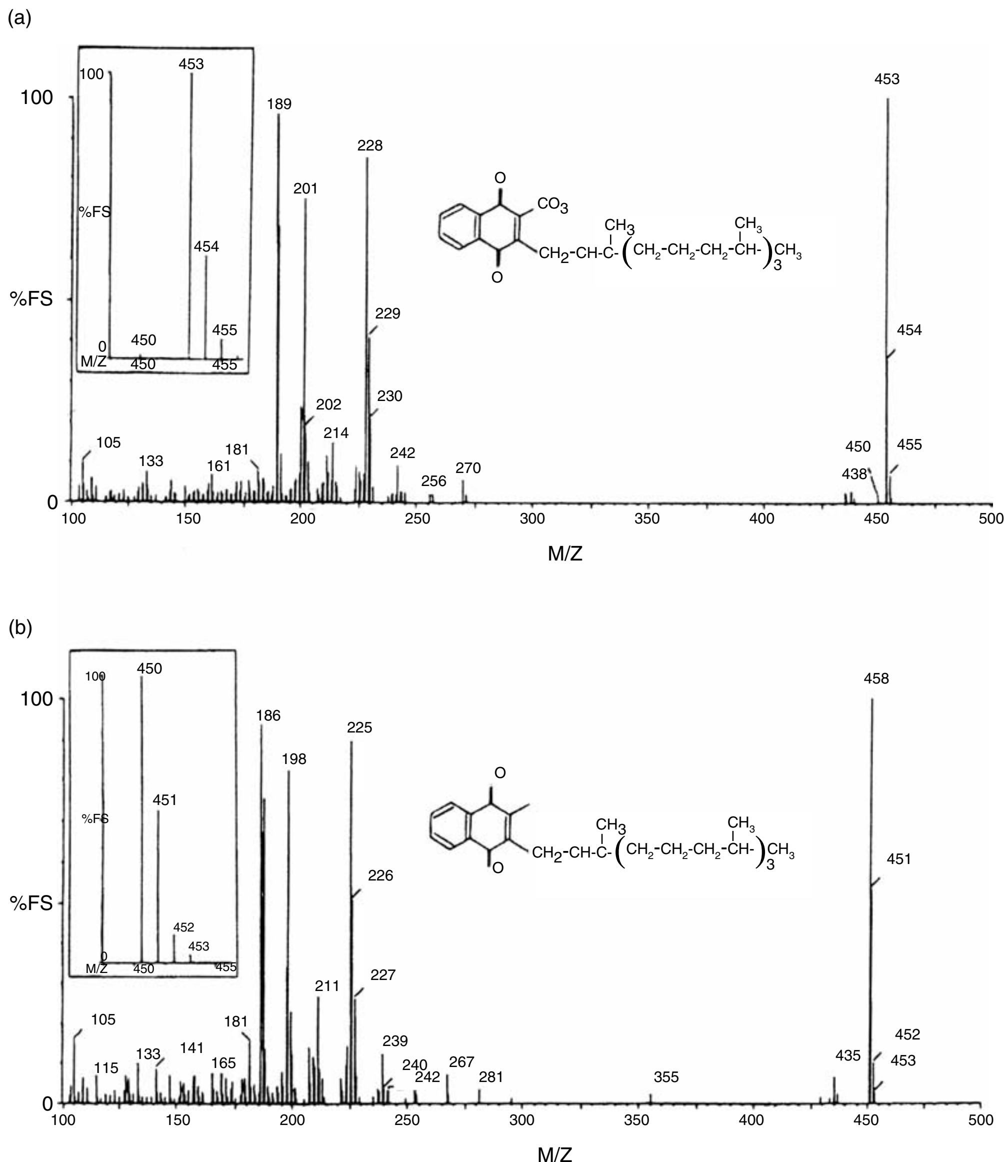

Figure 4.3 EI mass spectra, molecular structures, and isotopic composition of the molecular ion (inset) of unlabelled (b) and deuterium-labeled (a) vitamin $K_{1(20)}$. (Reproduced from Fauler, G., Leis, H., Schalamon, J., Muntean, W., and Gleispach, H., *J. Mass Spectrom.*, 31, 655, 1996. With permission.)

quinones to highly fluorescent hydroquinones. Fluorescence detection coupled to the resolution power of LC presented a great advance to the accurate determination of vitamin K in biologicals. Now LC-MS represents a significant further advancement and vitamin K can quantitated with a great deal of confidence.

4.3.2 Regulatory and handbook methods

Table 4.5 summarizes regulatory and handbook methods available for the analysis of vitamin K in pharmaceuticals and foods. Procedures for pharmaceuticals in the

Table 4.5 Regulatory and Handbook Methods for the Analysis of Vitamin K and Related Compounds

Source	Form	Methods and application	Approach	Most current cross-reference
***U.S. Pharmacopeia National Formulary*, 2006, USP 29/NF 24, Dietary Supplements Official Monograph**[61]				
1. Pages 2388–2390	Phylloquinone (K_1)	Phytonadione in oil-soluble vitamin capsules/ tablets	LC 254 nm	None
2. Pages 2391, 2395, 2398, 2411–2412	Phylloquinone (K_1)	Phytonadione in oil– and water-soluble vitamin capsules/tablets with or without minerals	LC 254 nm	None
3. Pages 1730–1731	Phylloquinone (K_1)	Phytonadione (NLT 97.0%, NMT 103.0%)	LC 254 nm	None
4. Pages 1731–1732	Phylloquinone (K_1)	Phytonadione injectable emulsion, tablets	LC 254 nm	None
5. Page 1336	Menadione (K_3)	Menadione injection (NLT 98.5%, NMT 101.0%)	Colorimetric 635 nm	None
***British Pharmacopoeia*, 2007**[62]				
1. Page 1332–1333	Menadione (K_3)	Vitamin K_3 (NLT 98.5%, NMT 101.0%)	Titration	None
***AOAC Official Methods of Analysis*, 18th ed., 2005**[63]				
1. 41.1.31	Menadione (K_3)	AOAC Official Method 974.30 Menadione sodium bisulfate (water-soluble vitamin K_3) in feed premixes	GC flame ionization	*J. Assoc. Off. Anal. Chem.*, 56, 1277, 1973[64]
2. 50.1.06	Phylloquinone (K_1)	AOAC Official Method 992.27 *trans*-Vitamin K_1 (phylloquinone) in Ready-To-Feed Milk-Based Infant Formula	LC 254 nm	*J. AOAC Int.*, 76, 1042, 1993;[65] *J. Assoc. Off. Anal. Chem.*, 68, 684, 1985[66]
3. 50.1.25	Phylloquinone	AOAC Official Method 999.15 Vitamin K in milk and infant formulas	LC Postcolumn reduction Fluorescence	*J. AOAC Int.*, 83, 121, 2000;[67] *Analyst*, 122, 465, 1997;[68] *J. AOAC Int.*, 85, 682, 2002[69]
***European Committee for Standardization*, 2003**[119]				
1. EN14148	Phylloquinone (K_1)	Foodstuffs—determination of vitamin K_1 by LC	LC Postcolumn reduction Fluorescence	*Analyst*, 122, 465, 1997;[68] *J. AOAC Int.*, 83, 121, 2000;[67] *J. AOAC Int.*, 85, 682, 2002;[69] *J. AOAC Int.*, 78, 719, 1995[84]
***Food Chemicals Codex*, 5th ed., 2004**[120]				
1. Pages 499–450	Phylloquinone (K_1)	Vitamin K_1 (NLT 97.0%, NMT 103.0%)	LC 254 nm	None

U.S. Pharmacopeia[61] and *British Pharmacopoeia*[62] use LC in place of older colorimetric or spectrophotometric methods. For food analysis, the AOAC *Official Methods of Analysis*[63] provides a GC procedure, Method 974.30 "Menadione Sodium Bisulfate in Food Premixes" for analysis of vitamin K_3 in feed premixes. For analysis of milk and infant formulas, AOAC Method 992.27 "*trans*-Vitamin K_1 (Phylloquinone) in Ready-To-Feed Milk-Based Infant Formula" was collaborated in 1993.[65] Method 992.27 follows the work of Hwang,[66] which used an open-column chromatographic cleanup step on silica. The method quantifies *trans*– and *cis*-isomers of vitamin K_1 by normal phase LC. A major deficiency of the method related to products formulated with corn oil in that interferences at 254 nm masked the *cis*-isomer peak. In addition, chromatograms of most samples tended to be complex because of lipid interferences. Overall, RSD_r values were unacceptably high at 20.9%.[65]

Method 992.27 was the only LC method provided by AOAC International for the analysis of vitamin K in infant formula before 2000 when Method 999.15 was collaborated.[67] Method 999.15 "Vitamin K in Milk and Infant Formulas" relies on accepted methods of vitamin K extraction from biological materials including digestion with lipase from *Candida cylindracea*, extraction of the digest with hexane, reversed-phase chromatography and fluorescent detection of the analytes after postcolumn reduction with zinc. General methodology is discussed more fully in Section 4.3.3. The procedure for method 999.15 follows earlier work on Indyk and Woolard[68] for quantitation of vitamin K_1 and menaquinone-4 (MK4, vitamin K_2) in milk and infant formula. Collaboration from data submitted by 33 internationally recognized laboratories showed the procedure to be highly reproducible with a RSD_R value of 6.53% and a HORRAT value of 0.33. Vitamin K_1 *cis*- and *trans*-isomers could be resolved with a C_{30} column.

Wollard et al.[69] expanded use of AOAC Method 999.15 to a wider range of foods with the C_{30} support. Figure 4.4 shows comparative resolution of vitamin K standards on C_{30} and C_{18} columns. Ability to resolve the *cis*- and *trans*-isomers is necessary to accurately assess

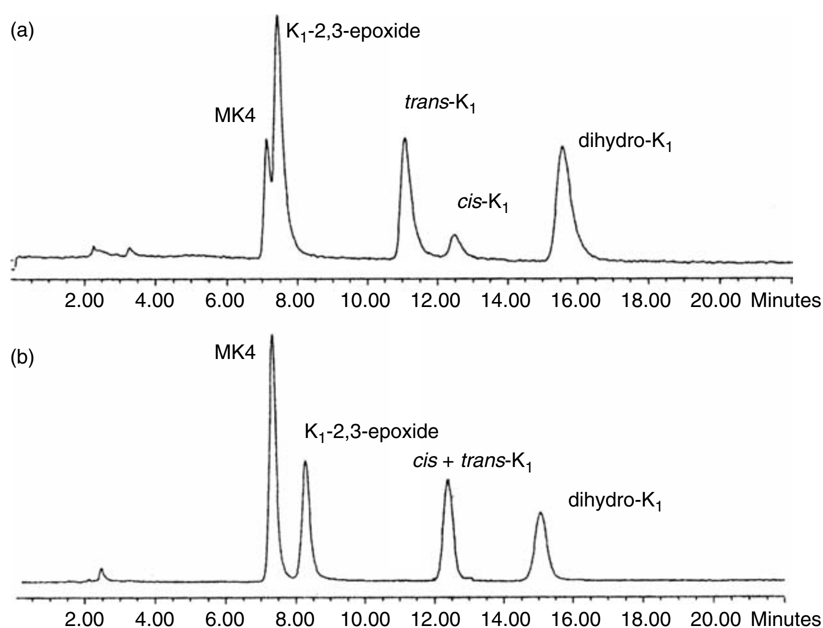

Figure 4.4 Chromatograms of canola oil on a YMC C_{30}, 3 µm, column (a) and a C_{18} column (b). Unidentified peak (µ). (Reproduced from Woollard, D. C., Indyk, H. E., Fong, B. Y., and Cook, K. K., *J. AOAC Int.*, 85, 682, 2002. With permission.)

the true levels of the biologically active *trans*-isomer of K_1. The added advantage of quantification of menaquinone makes the method valuable for general food analysis. Levels of *cis*-vitamin K_1, while generally low in most foods, contributed greatly to the total vitamin K_1 in some food samples (palmolein, 20.8%; canola oil, 19.2%; soybean oil, 12.5%; dried tomato soup, 10.6%; white bread, 11.5%). Details of the method are provided in Section 4.4.

In 2003, the European Committee for Standardization (CEN) made the procedure presented by the Indyk and Woollard group[67–69,84] a European Union National Standard—EN14148 "Foodstuffs—Determination of vitamin K by HPLC."[119] On the basis of work with the C_{30} column and its ability to resolve the inactive *cis*-K_1, the assay method was deemed applicable to all foods. EN14148 presents the vitamin K assay in a highly understandable format together with all supporting validation data.

4.3.3 Advances in analysis of vitamin K

4.3.3.1 Spectroscopic and electrochemical methods

Excellent spectroscopic methods exist for the analysis of vitamin K_3 (menadione) in pharmaceuticals and feeds using flow injection analysis and spectrophotometric, spectrofluorometric, or chemiluminescence detection. Electrochemical methods, while not as common as spectroscopic assays, are also available. Newer methods are summarized in Table 4.6.[70–78]

Spectroscopic methods for menadione and menadione sodium bisulfite, a water-soluble form of menadione, based on flow injection analysis are sensitive, reproducible, usually simple, and provide high sample throughput. The fluorometric assay published by Pérez-Ruiz et al.[73] provides a good example of methods available for vitamin K analysis. The procedure used online reduction of menadione in dodecylsulfate micelles by irradiation with UV light. The menadione was converted to its highly fluorescent dihydronaphtoquinone, which was quantified at Ex $\lambda = 340$ and Em $\lambda = 410$. The limit of detection was 0.18 ng mL,$^{-1}$ and the sample throughput was 90 h^{-1}.

Pérez-Ruiz et al.[78] adapted the procedure developed for menadione to the analysis of phylloquinone in fruit and vegetables. After extraction of the foods with hexane, sample extracts were treated by solid phase extraction on silica gel cartridges. Extractions were automated with direct injection of the sample extracts into the flow injection system. The detection limit of the assay was 0.05 µg mL^{-1} with sample throughput of 90 h.$^{-1}$ Results obtained with the online flow injection analysis closely compared to those determined by LC analysis for several fruits and vegetables.

4.3.3.2 High performance liquid chromatography

4.3.3.2.1 Extraction procedures for vitamin K analysis by liquid chromatography. The instability of vitamin K under alkaline conditions precludes the use of saponification for sample extraction. The necessity to remove other lipid components from vitamin K extracts before LC quantitation was a hindrance to early attempts to assay the vitamin from complex, fat-containing matrices. Extraction with water-soluble organic solvents such as ethanol, isopropanol, and acetonitrile to denature proteins followed by partitioning with nonpolar solvents efficiently can be used to extract vitamin K from serum. However, when used with more complex matrices, a total fat extract is obtained that must be extensively fractionated to eliminate the fat before determinative chromatography. Chromatograms obtained by LC and UV detection were often complex with poor resolution of vitamin K compounds from chromatographic interferences due to lipid components.

Method summaries are presented in Table 4.7 that trace the development of LC methodology for vitamin K analysis in foods and biologicals to its current stage. Development of extraction procedures that efficiently removed fat from the initial homogenate or extract

Table 4.6 Application of Spectroscopic and Electrochemical Methods to Analysis of Vitamin K

Matrix/analyte	Description	Results	References
	Pharmaceuticals		
Spectrophotometric			
Capsules/MSB	Vitamin K_3 (menadione sodium hydrogen sulphite) was indrectly determined by the reduction of iron (III) to iron (II) with blue color formation by the reaction of iron (II) with 2,4,6-(2′-pyridyl)-s-triazine (TPTZ). Color development was monitored at 595 nm	FIA using the flue colored iron (II)-TPTZ complex was linear at 2–10 μg mL^{-1} vitamin K_3. The method was applied to MSB in capsules	J. *Chem. Soc. Pak.*, 24, 119, 2002[70]
Injectables and tablets/ vitamin K_3, MSB	Reaction of vitamin K_3 and MSB with 3-methyl-2-benzothiazolineone hydrazone hydrochloride (MBTH) to yield a blue complex with maximum absorption at 625 nm and with resorcinol to yield a red complex with maximum absorption at 520 nm were studied for application to assay of pharmaceuticals	Formation of the MBTH and resorcinol coplexes provided rapid assays (<5 min) that were sensitive and reproducible	J. *Pharm. Biomed.* Anal., 28, 161, 2002[71]
Spectrofluorometric			
Injectables/MSB	Photochemically induced fluorescence of MSB in the presence of acetone and sodium sulphite produced a compound with Ex λ = 336, Em λ = 459	FIA of the fluorescence enhanced reaction product provided an assay with throughput of 90 h^{-1}. DL = 0.38 μg L^{-1}. The assay was successfully applied to MSB injectables	*Anal. Sci.*, 17, 1209, 2001[72]
Tablets, injectables/ vitamin K_3	On-line reduction of vitamin K_3 in SDS micelles by UV irradiation induced fluorescence at Ex λ = 340, Em λ = 410	The FIA had throughput of 90 h^{-1}. DL = 0.18 ng mL^{-1}	*Anal. Chim. Acta*, 514, 259, 2004[73]
Chemiluminscence			
Tablets, injectables/ vitamin K_3	The photooxidation of EtOH sensitized by vitamin K_3 to yield H_2O_2 was monitored with the hematin catalyzed chemiluminescent reaction with luminol	The FIA had throughput of 30 h^{-1}. DL = 2.03×10^{-1} mol L^{-1}. RSD = 1.2%. %Recovery = 95–105	*Analyst*, 124, 197, 1999[74]
Tablets, injectables/ MSB	A chemilumescence sensor was developed to assay MSB. The sensor is based on the autooxidation of bisulfite liberated from MSB in alkaline media in the presence of Tween 80 sensitized by Rhodamine 6G immobilized on a cation-exchange column	The linear range of the sensor was 0.5–1.0 μg mL^{-1} and the DL was 2.6 μg L^{-1}. The sensor could be used for >250 assays and was applicable to tablets and injectables. %RSD = 3.4	*Anal. Sci.*, 15, 1227, 1999[75]

Electrochemical			
Injectables/ vitamin K_3	The polarographic catalytic wave of vitamin K_3 in the presence of KIO_3 had a peak potential of –0.95 V versus SCE. The second order derivative peak current was directly proportional to the vitamin K_3 concentration	The polarographic catalytic wave of vitamin K_3 resulting from the chemical regeneration of the carbonyl group of vitamin K_3 because of the oxidation of the free radical intermediates of the vitamin by KIO_3 enhanced the maximum current of the first charge transfer to a level 10 times higher than the corresponding reduction wave in the absence of KIO_3. DL = 2.0×10^{-8} mol L^{-1}	*Anal. Lett.*, 34, 1677, 2001[76]
Human plasma/ vitamin K_3	Polyvinyl chloride matrix membrane sensors using ion association complexes of vitamin K_3 with bathophenanthroline nickel (II) and iron (II) as electroactive materials were produced for potentiometric batch or FIA of vitamin K_3 in human serum	The plasticized sensors provided a rapid method for direct assay of vitamin K_3 in serum without derivatization or extraction. Assays are completed in <5 min with a high degree of accuracy	*Mikrochim. Acta*, 138, 53, 2002[77]
	Food		
Spectrofluorometric			
Fruits and vegetables/ vitamin K_1	Vitamin K (phylloquinone in fruits and vegetables was assayed by online reduction of vitamin K_1 in SDS micelles in the presence of MeOH under UV irradiation fluorescent reduced phylloquinone allowed fluorescent monitoring at Ex $\lambda = 346$, Em $\lambda = 425$	Throughput of the FIA system was 90 h^{-1}. DL = 0.05 μg mL^{-1}. Application to fruits and vegetables required SPE cleanup with silica cartridges (Speed, Applied Separations). %Recovery was 97 ± 2	*Anal. Bioanal. Chem.*, 384, 280, 2006[78]

Table 4.7 LC And LC-MS Methods for the Analysis of Vitamin K in Foods, Pharmaceuticals, Feed, and Biologicals

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods					
Infant formula/ K_1, other fat-soluble vitamins	Add cholesterol phenyl acetate (IS). Lipase hydrolysis, add NH_4OH, EtOH, extract w/pentane	Two Zorbax-ODS in series, 25 cm × 4.6 mm Mobile phase—gradient MeOH–EtOAC–MeCN Variable flow rate	256 nm	—	*Anal. Chem.*, 52, 610, 1980[79]
Infant formula/K_1	Lipase hydrolysis. Add NH_4OH, EtOH, extract w/pentane	μBondapak C_{18}, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:MeCN:THF:water (39:39:16:6) Flow rate—1.5 mL min^{-1}	254 nm	%Recovery—84–103	*J. Assoc. Off. Anal.* Chem., 66, 1063, 1983[80]
Infant formula/ K_1, other fat-soluble vitamins	Add NH_4OH amd MeOH. Extract w/CH_2Cl_2:iOct (2:1). Evaporate, redissolve in iOct w/0.01% IPA. Cleanup: Silica open column	Apex Silica or Spherisorb ODS, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic NP—iOct:CH_2Cl^2:IPA (70:30:0.02) RP—THF:MeOH:water (27:67:6) Flow rate—1 mL min^{-1}	254 nm	QL—2 μg L^{-1} as fed %RSD—4.1 %Recovery—86–104	*J. Assoc. Off. Anal. Chem.*, 68, 684, 1985[66]
Vitamin premix/K_1	Add DMSO, heat at 65°C in ultrasonic bath, 30 min. Extract w/Hex, evaporate. Redissolve in IP for reversed-phase LC. Inject Hex directly for normal-phase LC	RP—Rad-Pak C_{18}, 5 μm NP—Silica Rad-Pak, 5 μm, 10 cm × 0.8 mm Mobile phase—isocratic RP—96% MeOH in water NP—Hex:IPA (99.1:0.1) Flow rate—1.0–2.5 mL min^{-1}	254 nm and Fluorescence Ex λ = 325 Em λ = 420	DL (on-column)—10 ng by fluorescence	J. *Micronutr. Anal.*, 4, 61, 1988[81]
Infant formula/ K_1, D_3	Extract lipids w/CH_2Cl_2 and IPA, dry w/$MgSO_4$. Cleanup: GPC—4 μStryagel 100 Å in series. μBondapak/NH_2, 25 cm × 4.6 mm	Zorbax ODS, 6 μm, 25 cm × 4.6 mm Mobile phase—isocratic CH_2Cl_2:MeCN:MeOH (300:700:2) Flow rate—1 mL min^{-1}	254 nm	%CV—2.8	*J. Food Compos. Anal.*,2, 140, 1989[82]

Milk, human/K_1	Add MK-5 (IS) in EtOH, albumin, sodium taurcholate, $CaCl_2$, NaCl, sonicate. Lipase hydrolysis. Add NH_4OH, EtOH extract w/Hex Cleanup: R Sil adsorption column	RoSil, C_{18}, HL, 5 μm, 15 cm × 3.2 mm Mobile phase—isocratic MeOH:EtOAC (96:4) containing tetramethyl ammonium octahydridotriborate Flow rate—0.7 mL min^{-1}	$K_{1\,(20)}$ and $K_{1\,(25)}$ reduced postcolumn Fluorescence Ex λ = 325 Em λ = 430	QL—35 ng L^{-1} $\%CV_{Intra}$—5.2 $\%CV_{Inter}$—5.8 %Recovery—62.5	*Clin. Chem.*, 38, 1743, 1992[83]
Infant formula/K_1	Add NH_4OH, MeOH, CH_2Cl_2: iOct mixture (2:1). Collect organic layer, evaporate. Cleanup: Sillica over-layered w/sodium sulfate	Apex I, silica, 5 μm Mobile phase—isocratic CH_2Cl_2:iOct (30:70) containing 0.02% IPA Flow rate—1 mL min^{-1}	254 nm	$\%RSD_r$—3.2 $\%RSD_R$—16.0	*J. AOAC Int.*, 76, 1042, 1993[65]
Infant formula, milk, bovine/K_1	Lipase hydrolysis. Add EtOH: MeOH (95:5), potassium carbonate and cholesteryl phenylacetate (IS), extract w/Hex Clean-up: semiprep LC, Resolve silica (8 × 10) radial compression	Resolve C_{18}, 5 μm, 8 × 10 RCM Mobile phase—isocratic MeOH:IPA:EtOAC:water (450:350:145:135) Flow rate—2 mL min^{-1}	Dual wavelength 269 nm 277 nm	DL (on-column)—1 ng QL—0.1–0.5 μg 100 g^{-1} %Recovery—88.9 $\%RSD_r$—1.5 $\%RSD_R$—2.8	*J. AOAC Int.*, 78, 719, 1995;[84] *Food Chem.*, 54, 403, 1995[85]
Various foods/K_1	Add dihydro-K1 (IS). Homogenize, extract w/Hex. Evaporate, redissolve in Hex. Add equal volume of MeOH:water (9:1) to Hex extract, mix, centrifuge, remove upper Hex, layer. Evaporate to dryness, dissolve residue in mobile phase	Hypersil ODS, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic CH_2Cl_2:MeOH (100:900) containing 10 mM ZnCl, 5 mM HAC, 5 mM NaOAC per 1000 mL Flow rate—1 mL min^{-1}	Postcolumn derivatization Fluorescence Ex λ = 243 Em λ = 430	%CV (within run)—3–98 %Recovery—95–101	*Food Chem.*, 56, 87, 1996[86] & 68, 219, 2000[87] & 74, 275, 2001[88]
Infant formula, milk, bovine/ K_1, K_2, dihydrophyllo-quinone	Dissolve or dilute 1 g powder or 10 g fluid milk to 15 mL w/water. Add 5 mL phosphate buffer, pH 7.9–8.0. Add lipase, i ncubate, 37°C, 2 h. Add 10 mL reagent alcohol and 1 g potassium carbonate. Extract w/Hex. Evaporate, redissolve in MeOH	Resolve C_{18}, 5 μm, 8 × 10 RCM Mobile phase—isocratic MeOH:IPA:EtOAC:water (450:350:145:135) Flow rate—2 mL min^{-1}	Dual wavelength 269 nm 277 nm	DL (on-column)—30 pg %Recovery > 98 $\%RSD_R$—2.35 for K_1 and 2.32 for MK-4	*Analyst*, 122, 465, 1997[68]

Continued

Table 4.7 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods (*Continued*)					
Vegetable, juice, milk, tea, coffee, spinach, bread, ground beef, edible oils,/K_1	Add dihydrophylloquinone (IS), IPA:Hex (3:2), water, sonicate, vortex, centrifuge. Evaporate Hex layer. Redissolve in Hex (whole milk requires additional Hex partitioning step). Cleanup: SPE	Hypersil ODS, 3 μm, 15 cm × 4.6 mm Mobile phase—isocratic MeOH:CH_2Cl_2 (90:10) containing 10 mM ZnCl, 5 mM HAC, 5 mM NaOAC per 1000 mL Flow rate—1 mL min^{-1}	Postcolumn derivatization Fluorescence Ex λ = 244 Em λ = 418	%CV—12.6–13.8	*J. Agric. Food Chem.*, 40, 1869, 1992[11] & 40, 1874, 1992[12] & 42, 295, 1994[14] & 43, 1574, 1995;[16] *Meth. Enzymol.*, 282, 446, 1997;[21] *J. Am. Diet. Assoc.*, 95, 82, 1995[15] & 103, 1650, 2003;[29] *Lipids*, 31, 715, 1996[20] & 31, 209, 1996[19]
Oil, margarine, butter, animal products/ K_1, MK-4 to MK-10	Oil—add MK-4 (IS) to 0.5–1 g samples. Dilute to 10 mL w/Hex. Butter, margarine, fish & meat products—extract w/Hex and IPA: Hex (1:1); dairy products—acid hydrolysis; fat content >10% - lipase hydrolysis. Cleanup: semiprep LC μPorasil, 30 cm × 3.9 mm, 5 μm	Vydac 205TP54, 25 cm × 4.6 mm, 5 μm Mobile phase—isocratic 95% MeOH:0.025 M NaOAC, pH 3.0 Flow rate—1 mL min^{-1}	EC—Redox Mode +1.1 V (upstream), 0V (downstream)	DL (pg) K_1—50 MK-4—20 %CV—0.1–14.3 %Recovery—78–98	*Food Chem.*, 59, 473, 1997[89] & 64, 411, 1999[90] & 68, 223, 2000;[91] *J. Agric. Food Chem.*, 48, 6325, 2000[92]
Various foods/K_1, MK-4, dihydrophylloquinone	Extract samples w/Hex. For fat-free samples—mix sample w/water and extract w/IPA: Hex (3:2), evaporate the upper layer to dryness, redissolve in Hex. Clean-up: SPE silica	BDS Hypersil C_{18}, 5 μm, 15 cm × 3 mm Mobile phase—gradient MeOH (containing 10 mM $ZnCl_2$, 5 mM HAC and NaOAC)—CH_2Cl_2 Flow rate—gradient	Postcolumn derivatization Fluorescence Ex λ = 244 Em λ = 430	LOD—14 pg $injection^{-1}$	*JAOCS*, 79, 641, 2002;[27] *J. Food Compos. Anal.*, 16, 595, 2003[28] & 17, 379, 2004[30] & 18, 751, 2005;[32] *J. Agric. Food Chem.*, 54, 463, 2006;[34] *J. Food Sci.*, 71, S66, 2006[35]

Various foods/ K_1, dihydro-phylloquinone	Enzymatic extraction (EE)—Add phosphate buffer and lipase to the samples, vortex, incubate w/shaking, 37°C, 2 h. Cool, add reagent alcohol, potassium carbonate. Extract w/Hex. Evaporate Hex layer, redisslove in MeOH Direct extraction (DE)—homogenize sample w/Hex, filter, evaporate	YMC C_{30}, 3 µm, 25 cm × 4.6 mm Mobile phase—isocratic $MeOH:CH_2Cl_2$ (90:10) containing 10 mM ZnCl, 5 mM HAC, 5 mM NaOAC per 1000 mL Flow rate—0.8 mL min^{-1}	Fluorescence Ex λ = 243 Em λ = 430	%Recovery 82–100 by EE 93–99 by DE	*Food Chem.*, 67, 79, 1999;[93] *J. AOAC Int.*, 85, 682, 2002[69]
LC-MS					
Vegetables/K_1	Sonicate sample w/MeOH, centrifuge. Saponify the extract w/sodium carbonate, 80°C, 1 h. Extract w/Hex, 2×. Evaporate the Hex layer to dryness. Redissolve in MeOH	LiChrosorb RP-8, 10 µm, 25 cm 4.6 mm Mobile phase—isocratic, 100% MeOH Flow rate—0.6 mL min^{-1}	PB MS-CI, negative mode w/SIM Or UV 247 nm	DL (on-column)—2 ng	*Fresenius J. Anal. Chem.*, 355, 48, 1996[94]
Feeds					
Premixes, animal feed/K_1	Add carbozol (IS), $CHCl_3$, NH_4OH: celite sodium sulfate. Mix, 30 min, centrifuge. Dilute or concentrate $CHCl_3$ extract	LiChrosorb, Si 60, 5 µm, 25 cm × 3.2 mm Mobile phase—isocratic THF:Hex (5:95) or $CHCl_3$: Hex (4:96) Flow rate—1 mL min^{-1}	251 nm	DL—0.02 µg g^{-1}	*Int. J. Vit. Nutr. Res.*, 52, 248, 1982[95]
Premixes, animal feed/K_3 (MSB)	Add water:EtOH (6:4), mix 10 min. Add 10% tannin solution, mix 1 min. Centrifuge supernatant. Add Hex and 10% sodium carbonate. Collect Hex layer, evaporate	ODS—Hypersil, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic EtOH:water (6:4) Flow rate—0.6 mL min^{-1}	Postcolumn reduction Fluorescence Ex λ = 325 Em λ = 425	QL—0.02 µg g^{-1}	*J. Chromatogr.*, 301, 441, 1984[96]

Continued

Table 4.7 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Feeds (*Continued*)					
Rat chow/K_1	SPE CO_2, 8000 psi, 60°C, 20 min	μBondapak C_{18}, 10 μm, 15 cm × 3.9 mm Mobile phase—isocratic MeCN:0.025 M $NaClO_4$ (90:10) Flow rate—2 mL min^{-1}	EC reductive mode Ag electrode at –0.75 V versus calomel electrode	DL (at detector)—125 pg %Recovery—90.5 QL (product)—20 μg g^{-1}	*J. Chromatogr. Sci.*, 26, 458, 1988[97]
Animal feeds/K_1	Add CHCl3, NH4OH, Celite-sodium sulfate (3:10), shake 20 min. Neutralize w/HAC. Centrifuge, dilute, or concentrate $CHCl_3$ extract. Extraction based on reference	LiChrosorb Si 60, 5 μm, 25 cm × 4 mm Mobile phase—isocratic CH_2Cl_2 (100%) Flow rate—1.8 mL min^{-1}	251 nm	DL (on-column)—2.5 ng %Recovery—93–97	J. Assoc. Off. Anal. Chem., 71, 826, 1988[98]
Animal feeds/K_3 (MSB)	Extract w/water:MeOH (60:40). Shake 30 min. Centrifuge, add 5% sodium carbonate, extract w/*n*-pentane. Evaporate, redissolve in MeOH	Supelcosil LC-18, 5 μm, 25 cm × 4 mm Mobile phase—isocratic MeOH:water (75:25) Flow rate—0.9 mL min^{-1}	Postcolumn reduction Fluorescence Ex λ = 325 Em λ = 425	QL—20 μg kg^{-1} %Recovery > 90	*J. Chromatogr.*, 472, 371, 1989[99]
Animal feeds/K_1, K_2, (MK-4) K_3	Add $CHCl_3$, shake 3 min. Add celite:sodium sulfate (3:10), shake 30 min. Neutralize w/HAC, centrifuge, filter $CHCl_3$ extract layer	LiChrosorb Si 60, 5 μm, 25 cm × 4 mm Mobile phase—isocratic Hex:CH_2Cl_2 (1:1) Flow rate—1 mL min^{-1}	264 nm	QL—100 ng g^{-1}	*Anal. Proc.*, 30, 266, 1993[100]
Pharmaceuticals					
Intravenous lipid emulsion/ K_1	Add dihydro-K_1 (IS). Extract w/IPA:Hex (3:2), centrifuge. Evaporate Hex. Cleanup: a. SPE silica gel. b. Liquid phase reductive extraction	Hypersil ODS Mobile phase—isocratic MeOH:CH_2Cl_2 (85:15) containing 10 mM $ZnCl_2$, 5 mM HAC and 5 mM NaOAC Flow rate—not specified	Postcolumn reduction Fluorescence Ex λ = 244 Em λ = 418	—	*JPEN*. 17, 142, 1993[101]

Intravenous fat emulsion, soybean oil/K_1	Add $K_{1(25)}$ (IS), 0.9% NaCl, EtOH, extract w/Hex Cleanup: Sep-Pak silica	XL 3 μm, Octyl cartridge, 7 cm × 4.7 mm Mobile phase—isocratic MeCN:EtOH (95:5) containing 0.005 M sodium perchlorate Flow rate—0.8 mL min^{-1}	EC reduction followed by fluorescence detection	QL—2 ng K_1 g^{-1} %RSD—4–7	*J. Chromatogr. A*, 664, 189, 1994[102]
Biologicals					
Plasma/K_1	Add $K_{1(25)}$ (IS), EtOH, extract w/Hex. Evaporate, redissolve Hex:DIPE (98.5:1.5) Cleanup: semiprep silica column, RoSIL 5 μm. Collect K fraction, concentrate	RSIL C_{18} HL, 5 μm, 15 cm × 3.2 mm Mobile phase—isocratic MeOH:EtOAC (96:4) Flow rate—0.7 mL min^{-1}	Postcolumn reduction Fluorescence Ex λ = 325 Em λ = 430	QL—50 pg mL^{-1}	*Anal. Biochem.*, 158, 257, 1986[103]
Plasma/K_1	Add dihydro-K_1 (IS), EtOH, extract w/Hex. Evaporate. Clean-up: (a) Sep-Pak silica; (b) Reductive extraction; (c) Reconversion of K_1—hydroquinone to K_1	Hypersil-ODS, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic CH_2Cl_2:MeOH (8:2) containing $ZnCl_2$, sodium acetate and HAC Flow rate—1 mL min^{-1}	Postcolumn reduction Fluorescence Ex λ = 325 Em λ = 430	QL—0.05 μg L^{-1} %CV < 10	*Clin. Chem.*, 32, 1925, 1986[104]
Human intestinal contents/K_1, MK-4 to MK-10	Add water:CHCl3:MeOH (10:12.5:25). Centrifuge. Add $CHCl_3$ and water to supernatant. Evaporate lower organic layer, redissolve in Hex. Cleanup: Sep-Pak silica	Novapak C_{18}, 15 cm × 3.9 mm Mobile phase—isocratic EtOH:water (95:5) Flow rate—0.7 mL min^{-1}	Postcolumn reduction Fluorescence Ex λ = 320 Em λ = 430	QL—0.02–0.05 μg g^{-1}	*Am. J. Gastroenterol.*, 87, 311, 1992;[105] *Meth. Enzymol.*, 282, 457, 1997[106]

Continued

Table 4.7 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Biologicals (*Continued*)					
Rat tissue/K_1, MK-4, MK-6, MK-7, MK-8, MK-9	Add water, EtOH, dihydro-vitamin K_1 (IS). Extract w/Hex. Evaporate, redissolve in Hex Cleanup: Silica 60	Chromospher C_{18}, 10 cm × 3 mm Mobile phase—isocratic MeOH:IPA:water (450:50:7) containing tetramethyl ammonium octahydrotriborate	Postcolumn reduction Fluorescence Ex λ = 244 Em λ = 430	QL—0.02–0.05 μg g^{-1}	*Br. J. Nutr.*, 72, 415, 1994[107] & 75, 121, 1996[108]
Serum/K_1	To 500 mL serum, add 1 mL 1 ng mL^{-1} K_2 (IS) in IPA, vortex. Extract 3 × w/2 mL Hex Centrifuge. Dry under nitrogen. Dissolve residue in Hex. Clean-up: SPE silica	Vydac 201TP54, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic EtOH:MeOH (40:60) Flow rate—1 mL min^{-1}	Postcolumn reduction Fluorescence Ex λ = 242 Em λ = 430	DL (on-column) — 20 pg %Recovery—70	*J. Chromatogr. B*, 670, 209, 1995[109]
Plasma/K_1	Add $K_{1\,(25)}$ (IS) EtOH to 0.5 mL plasma, vortex. Add water, Hex, mix. Centrifuge, evaporate Hex layer, redissolve in IPA. Cleanup: SPE C_{18}	BDS-Hypersil, 3 μm, 15 cm × 3.0 mm Mobile phase—isocratic CH_2Cl_2:MeOH (10:90) containing $ZnCl_2$, HAC and NaOAC Flow rate—0.6 mL min^{-1}	Postcolumn reduction Fluorescence Ex λ = 244 Em λ = 418	%Recovery (IS)—75 QL—15 pg mL^{-1}	*Meth. Enzymol.*, 282, 408, 1997[110]
Plasma/K_1, K_1, 2, 3 epoxide	Add MK-6 (IS), 3.8 mL EtOH, and 12 mL Hex to 0.5 mL plasma. Vortex, centrifuge. Evaporate Hex layer to dryness. Clean-up: SPE on silica followed by SPE on C_{18}	BDS-Hypersil, 5μm, 25 cm × 2.1 mm Mobile phase—isocratic CH_2Cl_2:MeOH (10:90) containing $ZnCl_2$, HAC and NaOAC Flow rate gradient	Postcolumn reduction Fluorescence Ex λ = 244 Em λ = 418	DL (pg) on-column K_1—4 K_1 epoxide—5	*Meth. Enzymol.*, 282, 421, 1997[111]
Plasma/K_1	Add MK-6 (IS), precipitate proteins w/EtOH. Extract w/Hex Cleanup: semiprep LC, Spherisorb CN Nitrile, 5 μm	Exsil C_{18}, 5 μm, 25 cm × 5 mm Mobile phase—isocratic 0.05 M NaOAC, pH 3.0, in MeOH Flow rate—1 mL min^{-1}	Dual electrode coulometric—1.2–1.6 V, amperometric electrode at + 0.15-+ 0.2 V	DL—2–5 ng L^{-1} %RSD—1.6–4.1 Intra-assay precision—9.8–11.3%	*Meth. Enzymol.*, 282, 421, 1997[111]

Rat tissues/ *trans-*, *cis*-K_1	Homogenize sample (0.5–1g) w/2 mL EtOH and 10 mL CH_2Cl_2 containing BHT. Filter, evaporate 8 mL extract to dryness. Redissolve in THF: MeOH (1:1) containing BHT	RP: YMC C_{30}, 3 μm, 25 cm × 4.6 mm Mobile phase—isocratic, 100% MeOH Flow rate—1 mL min^{-1} or NP: Partisil, 10 μm, 25 cm × 4.6 mm Mobile phase—isocratic Hex:IPA (99.9:0.1) Flow rate—2 mL min^{-1}	Postcolumn reduction Fluorescence Ex λ = 243 Em λ = 430 or UV 254 nm	%Recovery > 86	*J. AOAC Int.*, 85, 832, 2002[112]
Human serum/K_1, MK-4, MK-5, MK-6, MK-7	Add 2 mL EtOH, 8 mL Hex to 0.5 mL serum. Vortex, centrifuge. Evaporate Hex layer to dryness. Redissolve in EtOH. Cleanup: SPE, ODS	Shodex C_{18}, 25 cm × 4.6 mm Mobile phase—isocratic EtOH:MeOH (1:1) containing 0.025 M sodium perchlorate Flow rate—0.6 mL min^{-1}	ECD glass carbon +0.6 V versus Ag/AgCl	DL—2–10 pg %Recovery—86–91 $\%CV_{Intra}$—1.6–2.1 $\%CV_{Inter}$—2.4–3.6	*Nutrition*, 19, 661, 2003[113]
Plasma/K_1	Add IS, saline and EtOH, vortex. Extract w/Hex. Collect Hex layer Cleanup: SPE, silica	BDS-Hypersil, 3 μm, 15 cm × 3.2 mm Mobile phase—gradient CH_2Cl_2—MeOH containing $ZnCl_2$, HAC and NaOAC Flow rate—0.6 mL min^{-1}	Postcolumn reduction Fluorescence Ex λ = 244 Em λ = 430	—	*Clin. Chim. Acta*, 347, 199, 2004[58]
Plasma/K_1, K_2, MK-4, Mk-7	Dilute serum w/water (1:1). Extract w/EtOH and Hex. Collect Hex layer Cleanup: Sep-Pak silica	Capcell Pak C_{18} 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:water (95:5) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 320 for MK-4, 240 nm for the rest Em λ = 430	DL—2–4 pg %Recovery—92 %RSD—4.9–19.3	*J. Chromatogr. B*, 816, 41, 2005[114]
LC-MS					
Human osteoblasts/ MK-4, MK-4 epoxide	Extract cells w/MeOH. Add deuterated MK-4 (IS)	L-column ODS, 15 cm × 1.5 mm Mobile phase—isocratic EtOH:MeOH (80:20) Flow rate—0.15 mL min^{-1}	MS-APCI positive ion mode	%RSD < 10 DL (on-column)—50 pg for MK-4 epoxide	*Anal. Sci.*, 13, 67, 1997[115]
Plasma/ K_1, K_2, MK-4, Mk-7	Dilute serum w/water (1:1). Extract w/EtOH and Hex Collect Hex layer. Cleanup: Sep-Pak silica	Capcell Pak C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient MeOH—0.1% HAC–EtOH Flow rate—1 mL min^{-1}	MS-APCI positive ion mode	—	*Anal. Chem.*, 77, 757, 2005[116]

were necessary to further the application of LC to complex matrices. In 1980, Barnett et al.[79] introduced the use of lipase hydrolysis as a primary extraction step. These investigators used the nonspecific lipase from the yeast *Candida cyclindraccae* to hydrolyze lipids in infant formula and dairy products as the initial step in the extraction of fat-soluble vitamins. Fatty acid hydrolyzates were then partitioned with pentane to yield an extract suitable for reversed-phase chromatography with detection at 265 nm. Barnett et al.[79] lipase hydrolysis procedure has been incorporated into many published procedures for vitamin K analysis of infant formulas, dairy products, and other foods (Table 4.7). A method developed by Bueno and Villalobos[80] modified the lipase hydrolysis procedure of Barnett et al.[79] in an attempt to reduce chromatographic interferences; however, UV absorbing interferences remained problematic.

Further, removal of lipid components is necessary after initial extraction of the lipid fraction containing vitamin K components. Most commonly, solid phase exraction (SPE) with silica cartridges is used. However, gel permeation chromatography and semipreparative LC also provide cleanup necessary for interference-free chromatograms. Piironen and colleagues[89–92] purified hexane extracts of many foods common to Finland by semipreparative LC on μPorasil. The cleanup was sufficient to permit use of electrochemical detection after reversed-phase chromatography on Vydac 201 TP54. This procedure is detailed in Section 4.4. Gel permeation was effectively used for extract cleanup by Landen et al.[82] Earlier methods by Haroon et al[117] and Indyk et al.[84] for analysis of bovine milk and infant formula and Lambert et al.[103] for serum analysis relied on semipreparative LC for cleanup of initial extracts. Cleanup procedures used for vitamin K extractions are fully discussed by Fauler et al.[53]

4.3.3.2.2 Chromatography parameters

4.3.3.2.2.1 Supports and mobile phases. Methods for vitamin K analysis have evolved through the combination of adsorption chromatography for extract cleanup and reversed-phase chromatography on C_{18} supports as the determinative step. Availability of the C_{30} polymeric support originally developed for improved resolution of carotenoid isomers (Chapter 1, Section 1.3.2.2.2.1) was used in the analysis of vitamin K in margarine,[93] milk and infant formulas,[67,69] other foods,[69] and rat tissue.[112] Use of the C_{30} support by Woollard et al.[69] resolved *cis*- and *trans*-isomers of vitamin K. Reversed-phase chromatography on C_{18} as provided in AOAC Official Method 999.15 collaborated by Indyk and Woollard[67] quantified vitamin K_1 as a single peak; thus, chromatography with the C_{30} support gives accurate data on the biologically active *trans*-isomer. The C_{30} support was first used by Cook et al.[93] to assay vitamin K_1 and dihydro-vitamin K_1 in margarine. Cook et al.[112] in a later study extended the C_{30} support to analysis of rat tissue.

Many mobile phase compositions have proven useful for vitamin K analysis with reversed-phase supports. Most common are isocratic separations using methanol modified with dichloromethane. Mobile-phase composition is dependent upon the detection mode. Electrochemical detection requires addition of an electrolyte such as sodium acetate or perchlorate in the mobile phase to support the conductivity. Postcolumn reduction of the quinones to the fluorescent hydroquinones requires the addition of zinc chloride when a postcolumn zinc metal reduction column is used.

4.3.3.2.2.2 Detection. Ultraviolet detection near absorption maxima can be used for high-potency samples including premixes, supplements, and vitamin pills. Use of UV fails as the complexity of the sample matrix increases and concentrations of vitamin K compounds decrease to clinical levels. Problems associated with UV interfering compounds present in lipid-containing extracts makes UV detection unworkable for many matrices.

Detection methods based on electrochemistry or reductive formation of fluorescent hydroquinones from the quinones were largely necessitated because of the lack of sensitivity and selectivity of UV detection.

Postcolumn reduction of vitamin K by zinc to form fluorescent hydroquinones represents a most significant advance for assay of vitamin K in biologicals. The approach utilizes techniques originally developed by Haroon et al.[104,117] for plasma and other biological samples. The method was based on postcolumn reduction of vitamin K (quinone) into fluorescent hydroquinones by use of a solid-phase reductive column packed with metallic zinc particles. The work followed studies by Langenberg and Tjaden[118] on application of an electrochemical detector for the reductive process followed by fluorescence detection for quantitation. Haroon's research showed that the solid-phase reactor offered many advantages over electrochemical reduction. Of significance, the zinc column increased reduction efficiency and simplified the instrumental setup compared to electrochemical reduction. The zinc postcolumn reduction inducement of fluorescence in vitamin K extracts forms the basis of many, highly specific assay methods for vitamin K. Methodology used by Sadowski and colleagues,[11–23] Booth and colleagues,[24–36] Cook and colleagues,[93–112] and Woollard and colleagues[67,69] for analysis of many foods use the solid-phase zinc reduction column introduced by Haroon et al.[104] These methods are detailed in Section 4.4. The sensitivity and specificity afforded by reductive formation of the fluorescent quinones makes this methodology preferential to many analytical approaches for vitamin K. Discussions by Davidson and Sadowski[110] and Booth and Sadowski[21] further detail chromatography systems using zinc reduction column methodology. Modification of the chromatography system permits simultaneous assay of the phylloquinone and phylloquinone 2,3-epoxide in plasma.[110]

Few research papers exist that have applied LC-MS instrumentation to vitamin K analysis. Careri et al.[94] first applied MS detection to the identification and quantitation of vitamin K in vegetables. Their work showed that the selectivity of MS detection could be readily used for food analysis. Particle beam mass spectra were obtained in electron impact and chemical ionization modes. LC-MS assays were completed with negative-ion chemical ionization by monitoring the molecular ion of vitamin K_1 atm/z 450.

Sano et al.[115] used LC-MS/MS-APCI for the analysis of MK-4 and a metabolite, MK-4 epoxide, with good success in 1997. More recently, Suhara et al.[116] quantified vitamin K_1, MK-4, and MK-7 in human plasma by LC-MS/MS-APCI. Their method included use of the internal standards MK-4-^{18}O, MK-7-^{18}O, and K_1-^{18}O that were synthesized in the authors' laboratory. They concluded that LC/APCI/MS/MS provides an "easy-to-use approach for quantitation of vitamin K analogues in human plasma without compromising assay sensitivity." Validation parameters for the method were excellent with low RSD% values for each analyte and high recoveries (98–102%).

4.3.3.2.2.3 Internal standards. The use of internal standards in the LC quantitation of vitamin K is encouraged because of the extensive cleanup of extracts that is required and, in the case of reductive formation of hydroquinones for fluorescence detection, the chance for incomplete conversion of the quinone to the fluorescent derivatives. Internal standards that are effective include vitamin $K_{1(25)}$, MK-5, MK-6, MK-7, 6-methyl-menquinone (MK-9) (Table 4.7). Dihydro-vitamin K_1 and vitamin K_1 epoxide quite frequently have been used as internal standards. Their use should be considered only after complete absence in the biological matrix is assured. Dihydro-vitamin K_1 has been documented to be a product of the hydrogenation of edible oils.[18–20] Therefore, use of dihydro-vitamin K_1 as an internal standard should be limited to biologicals and foods where its absence is documented. Stable isotope ^{18}O vitamin K homologues (K_1-^{18}O, MK-4-^{18}O, MK-7-^{18}O) were used by Kamao et al.[114] and Suhara et al.[116] in LC-APCI-MS/MS analysis.

4.4 *Method protocols*

Evaluation of an HPLC Method for the Determination of Phylloquinone (Vitamin K_1) in Various Food Matrices

Clin. Chem., 32, 1925, 1986;[104] *J. Agric. Food Chem.*, 40, 1869, 1992;[11] *J. Agric. Food Chem.*, 40, 1874, 1992;[12] *J. Agric. Food Chem.*, 42, 295, 1994[14]

Principle

After initial extraction in isopropanol and hexane, the food extracts were purified by solid-phase extraction on silica gel. The amount of K_1 was determined by LC by applying a postcolumn chemical reduction followed by fluorescence detection of the hydroquinone.

Chemicals

- Vitamin K_1 standard
- 2,3-dihydrophylloquinone—internal standard for vegetables, milk
- $K_{1(25)}$—internal standard for bread, beef
- Isopropanol
- Hexane
- Dichloromethane
- Methanol
- $ZnCl_2$
- Acetic acid
- Sodium acetate

Apparatus

- Liquid chromatograph
- Fluorescence detector
- Silica gel columns (3 mL) for solid-phase extraction
- Sonicator—Soniter-cell disrupter with tapered microtip
- Centrifugal evaporator

Procedure

For Vegetable Juice

- Weigh prepared sample in 50 mL centrifuge tubes
- Add internal standard
- Add 15 mL isopropanol:hexane (3:2, v/v) followed by 4 mL H_2O
- Sonicate, 30 s
- Aspirate hexane layer into culture tube
- Evaporate to dryness in a centrifugal evaporator
- Dissolve residue in 2 mL hexane

Solid-Phase Extraction on Silica Gel

- Precondition column by washing with 8 mL hexane/diethyl ether (93:3, v/v) and 8 mL hexane
- Apply 2 mL hexane extract
- Wash with 8 mL hexane
- Elute vitamin K_1 with 8 mL hexane:diethyl ether (93:3, v/v)
- Collect vitamin fraction in 16 × 100 mm screwcap culture tube
- Evaporate to dryness
- Reconstitute with 20 mL methylene chloride

- Add 180 mL methanol containing 10 mM $ZnCl_2$, 5 mM acetic acid, and 5 mM sodium acetate
- Inject 100 mL onto HPLC column

Chromatography (Figure 4.5)

Column	15.0 cm × 4.6 mm
Stationary phase	Hypersil ODS, 3 μm
Mobile phase	Methanol:methylene chloride (90:10, v/v). To each item, add 5 mL solution containing 2 M zinc chloride, 1 M acetic acid, and 1 M sodium acetate.

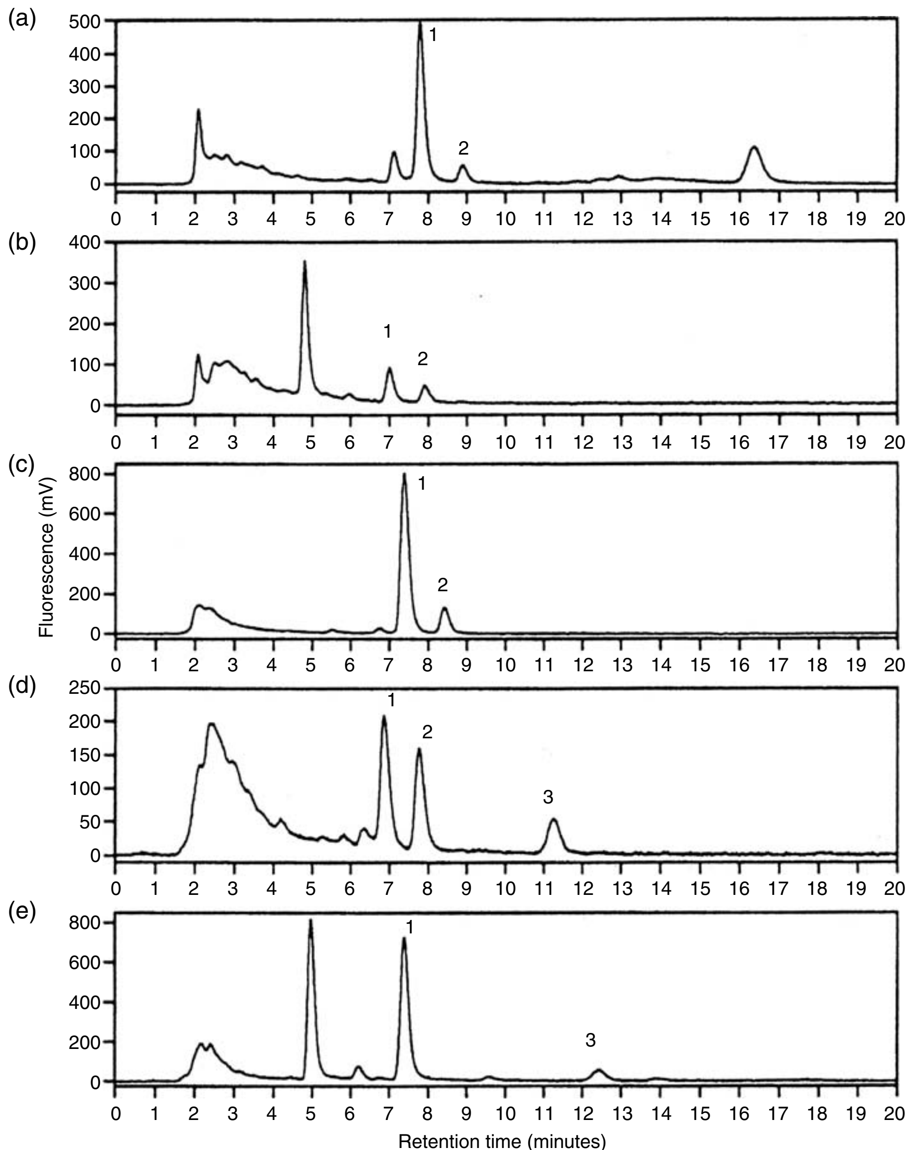

Figure 4.5 Chromatograms of extracts of vegetable juice (a), milk (b), spinach (c), bread (d), and beef (e). Peak 1—phylloquinone, Peak 2—dihydro-vitamin K_1, and Peak 3—Vitamin $K_{1(25)}$. (Reproduced from Booth, S. L., Davidson, K. W., and Sadowski, J. A., *J. Agric. Food Chem.*, 42, 295, 1994. With permission.)

Column temperature	Ambient
LC postcolumn reactor	2.0 cm × 50 mm packed with zinc metal
Flow rate	1 mL min^{-1}
Detection	Fluorescence Ex λ = 244, Em λ = 418
Calculation	Internal standard, peak area
Retention time	7.5 min

Vitamin K in Milk and Infant Formulas: Determination and Distribution of Phylloquinone and Menaquinone-4

Analyst, 122, 465, 1997[68]

Principle

Phylloquinone and menaquionone-4 are extracted by lipase digestion, protein denaturation with potassium carbonate, pH adjustment and alcohol, and hexane partitioning. Analytes are resolved by reversed-phase LC with fluorescence detection after zinc postcolumn reduction.

Chemicals

- Vitamin K_1 standard (USP)
- MK-4 standard
- Ethanol
- Methanol
- Sodium hydroxide
- Potassium carbonate
- Potassium phosphate
- Zinc chloride
- Sodium acetate
- Acetic acid
- Hexane
- Dichloromethane
- Lipase, Type VII (Sigma)
- Zinc powder

Apparatus

- Liquid chromatograph
- Fluorescence detector
- Stainless steel reductive column (20 × 4 mm)

Procedure

Extraction

- Weigh 1.0 g powdered milk or infant formula or 10 g fluid milk
- Add 15 mL warm H_2O (< 40°C)
- Add 5.0 mL phosphate buffer
- Add 1.0 g lipase
- Vortex and shake for 7 min
- Incubate at 37°C for 120 min (shaking each 20 min)
- Cool to ambient
- Add 10 mL reagent alcohol (EtOH + CH_3OH (95 + 5))
- Add 1.0 g K_2CO_3
- Mix

- Add 30.0 mL hexane
- Shake for 7 min
- Centrifuge at 1000 rpm min^{-1} for 10 min
- Transfer 0.5 mL hexane layer (supplemental formula) to glass vial
- Evaporate hexane
- Dissolve residue in 1.0 mL CH_3OH
- Filter (0.45 mm)

Chromatography

Column	Radial compression module, 10 cm × 8 mm
Stationary phase	C_{18} Resolve cartridge
Mobile phase	Dichloromethane 1 methanol (900 1 100) 1.5 mL methanolic solution of $ZnCl_2$ (1.37 g) 1 sodium acetate (0.41 g) 1 glacial acetic acid (0.3 g)
Flow rate	1.5 mL min^{-1}
LC postcolumn reactor	20 cm × 4 mm stainless steel column packed with Zn powder
Detection	Fluorescence, Ex λ = 243, Em λ = 430
Calculation	External standard, peak area

Determination of Vitamin K_1 in Foods by Liquid Chromatography with C_{30} Bonded-Phase Column

J. AOAC Int., 85, 682, 2002[69]

Principle

Vitamin K_1 was assayed by reversed-phase LC with a C_{30} column. Vitamin K_1 was reduced postcolumn to fluorescent hydroquinones.

Chemicals

- Lipase-L-1754, Type VII from *Candida rugosa* (Sigma)
- Papain-390303G from *Carica papaya* (BDH)
- $ZnCl_2$, Na acetate, glacial acetic acid, potassium carbonate, Zn powder
- Isopropanol, methanol, ethanol, hexane, dichloromethane, deionized water
- Standards—Vitamin K_1, MK4, MK5, MK6, MK7, MK8, MK9, K_1-2,3-epoxide

Apparatus

- LC, fluorescence detector
- Stainless steel reductor column, 20 mm × 4 mm
- Filtration device
- Rotary evaporator

Extraction—Milk powders, infant formula, cheese, baby foods, etc.

- Weigh sample into 100 mL Schott bottles
- Add 1.0–1.5 g lipase, 20 mL phosphate buffer (0.2 M)
- Incubate, 37°C, 2 h (maintain pH between 7.6 and 8.2)
- Cool, add 10 mL ethanol, 1 g potassium carbonate, 30 mL hexane
- Shake, 30 min
- Allow phase separation, overnight at 4°C or centrifuge
- Remove aliquot of upper organic phase, evaporate
- Dissolve residue in MeOH:IPA (1:1)

Chromatography

Column	YMC, 3 μm, 25 cm × 4.6 mm
Stationary phase	C_{30}
Mobile phase	Dissolve 0.41 g sodium acetate, 1.37 g zinc chloride and 0.30 g glacial acetic acid in 920 mL methanol. Add 80 mL dichloromethane.
Flow rate	1.5 mL min^{-1}
Detection	Fluorescence, Ex λ = 243, Em λ = 430
Calculation	External standard, peak area

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section two

Water-soluble vitamins

chapter five

Ascorbic acid: vitamin C

5.1 Review

Scurvy is the centuries old name for vitamin C deficiency. Like many other nutritional deficiencies, scurvy was recognized long before the active nutritional component was known and understood. With scurvy, associations were made with the curative and preventative effects of various foods. In 1747, James Lind, a British naval physician, documented the role of citrus fruits in curing British sailors suffering from scurvy. His work, representing the first controlled therapeutic medical trial, established citrus fruits as a curative food.[1] Essentially, no further advances were made until the disease was induced in guinea pigs in 1907. An animal assay based upon the guinea pig's response was developed in 1917 to test the antiscorbutic activity of foods. Ascorbic acid was isolated from natural sources, shown to be the antiscorbutic factor, and structurally characterized by the Szent-Györgyi group[2] and Haworth and King in the early 1930s. The vitamin was synthesized by Reichstein in 1933. In 1937, Szent-Györgyi and Haworth were awarded the Nobel Prize for their research on ascorbic acid.[3]

Olson[1] categorized scurvy symptoms as systemic, hemorrhagic, psychologic, secretory, neurologic, hematologic, and connective tissue related. Moderate deficiency can lead to fatigue and irritability. The symptoms are more fully described in Table 5.1. Serum and leukocyte ascorbic acid concentrations are frequently used to assess human ascorbic acid status.[4] Leukocyte concentrations are more reliable status indicators than serum, erythrocyte, or whole blood levels since short-term fluctuations in intake affect the leukocyte concentration to a lesser degree.[5] Urinary excretion decreases as intake decreases and is highly reflective of recent intake. Even with 24 h urine collections, urinary levels are not a sensitive index of status. Chemical and liquid chromatographic techniques provide highly reliable measures of ascorbic acid in clinical samples. Vitamin C status based on serum concentrations (μmol L^{-1}) are <11.4 (deficient), 11.4–23 (low), and >23 (adequate).[5]

Ascorbic acid is present in all animals and higher plants, but only humans and a few other vertebrates have specific requirements. Other species synthesize the compound. Vegetables and fruits are the primary dietary sources for the human. Citrus and various vegetables including peppers, tomatoes, potatoes, and leafy greens are excellent sources. Dairy products, meats, and cereal grains are poor sources of vitamin C. Some of the most concentrated food sources of vitamin C are given in Table 5.2. This data are derived from the United States Department of Agriculture Nutrient Database for Standard Reference, Release 19.[6]

Dietary Reference Intakes (DRI) for vitamin C are summarized in Table 5.3.[7] The Recommended Dietary Allowances (RDA) are 75 and 90 mg d^{-1} for adult women and men, respectively. This level is thought to maintain near-maximal vitamin C concentrations in

Table 5.1 Signs of Ascorbic Acid Deficiency

	Symptom
Systemic	Fatigue
	Lassitude
Hemorrhagic	Perifolicular hemorrhage
	Pinpoint red spots on the skin
	Bleeding gums
Psychologic	Depression
	Hypochondriasis
	Hysteria
Secretory	Dry skin } Sjögren's Syndrome
	Xerophthalmia } Sjögren's Syndrome
	Xerostomia } Sjögren's Syndrome
Vasomotor instability	Altered neurotrophic amine metabolism
Hematologic	Impaired iron absorption
	Impaired folate metabolism
Connective tissue	Scorbutic arthritis
	Impaired wound healing

Source: Olson, R. E., Olson, R. E., Water-soluble vitamins, In *Principles of Pharmacology*, Munson, P. L., Mueller, R. A. and Breese, G. R., eds., Chapman and Hall, New York, 1995, Chap. 59.

neutrophils and minimal urinary loss.[7] For nutritional labeling, the Reference Daily Intake (RDI) set by the Nutritional Labeling and Education Act of 1990 is 60 mg.[8]

L-Ascorbic acid is the active form of vitamin C. Its stereoisomer, D-isoascorbic acid, has only minimal biological activity. Biochemical functions are largely based upon the oxidation–reduction properties of the vitamin. The involvement of L-ascorbic acid in collagen synthesis is directly related to the etiology of scurvy. L-Ascorbic acid functions in hydroxylase reactions are active in the post-translational hydroxylation of collagen. It participates in collagen gene expression and synthesis of other corrective tissue components.[7] It acts as a cofactor in several enzymes as an electron donor. Such enzymes are Fe or Cu metalloenzymes, which participate in collagen hydroxylation and the biosynthesis of carnitine, hormones, and amino acids. Monoxygenases such as dopamine-β-hydroxylase incorporate one oxygen atom into a product with concomitant reduction of the other oxygen atom into water. Dopamine-β-hydroxylase functions in the conversion of dopamine into norepinephrine in catecholamine synthesis. In this class of enzymes, two substrates are oxidized and the term "mixed-function oxidase" is applicable. L-Ascorbic acid also functions with dioxygenases that require α-ketoglutarate as a cosubstrate. In these reactions, one oxygen atom is incorporated into succinate and one into the product. The procollagen proline hydroxylase conversion of proline into hydroxyproline characterizes this class of reactions. The coenzyme roles of L-ascorbic acid were discussed in detail by several authors.[9–11]

Vitamin C's role as an *in vivo* antioxidant has received much attention over the past decades. In this respect, it is included in the antioxidant complex including vitamin E, β-carotene, and other nonprovitamin A carotenoids such as lutein and lycopene, flavonoids, and selenium. While significant emphasis has been given to the antioxidant components of the diet, definitive clinical results are largely lacking. Like vitamin E, L-ascorbic acid is a primary defensive nutrient through its function as a free-radical scavenger of reactive oxygen and reactive nitrogen species. It can react in aqueous media against *in vivo* peroxidations, including quenching of singlet oxygen species. Therefore, it is considered to be a protective agent against atherosclerosis and a possible anticarcinogen. Interaction of ascorbic acid and vitamin E regenerates vitamin E in autoxidizing lipid systems. Ascorbic acid donates a hydrogen atom to tocopheroxyl radicals to regenerate vitamin E[12–17] (Figure 5.1).

Table 5.2 Vitamin C Content of Various Foods

Description	NDB no.	mg 100 g^{-1}	Description	NDB no.	mg 100 g^{-1}
Cereals ready-to-eat, KELLOGG, KELLOGG'S Complete Wheat Bran Flakes	08028	207	Strawberries, frozen, sweetened, sliced	09320	41
Cereals ready-to-eat, KELLOGG, KELLOGG'S PRODUCT 19	08058	204	Kale, cooked, boiled, drained, without salt	11234	41
Cereals ready-to-eat, GENERAL MILLS, TOTAL Corn Flakes	08246	200	Grapefruit juice, pink, raw	09404	38
Cereals ready-to-eat, GENERAL MILLS, Whole Grain TOTAL	08077	200	Melons, cantaloupe, raw	09181	37
Peppers, sweet, red, raw	11821	190	Pineapple, raw, all varieties	09266	36
Peppers, sweet, red, cooked, boiled, drained, without salt	11823	171	Carambola, (starfruit), raw	09060	34
Orange juice, frozen concentrate, unsweetened, undiluted	09214	138	Mangoes, raw	09176	28
Grapefruit juice, white frozen concentrate, unsweetened, undiluted	09125	120	Vegetable juice cocktail, canned	11578	28
Peaches, frozen, sliced, sweetened	09250	94	Mangoes, raw	09176	28
Kiwi fruit, (chinese gooseberries), fresh, raw	09148	93	Soup, tomato, canned, prepared with equal volume milk, commercial	06359	27
Broccoli, raw	11090	89	Sweet potato, canned, vacuum pack	11512	26
Grape juice, frozen concentrate, sweetened, undiluted, with added vitamin C	09136	83	Raspberries, raw	09302	26
Peppers, sweet, green, raw	11333	80	Nuts, chestnuts, european, roasted	12167	26
Broccoli, cooked, boiled, drained, without salt	11901	65	Mustard greens, cooked, boiled, drained, without salt	11271	25
Papayas, raw	09226	62	Kale, frozen, cooked, boiled, drained, without salt	11236	25
Strawberries, raw	09316	59	Lemon juice, canned or bottled	09153	25
Cabbage, red, raw	11112	57	Asparagus, frozen, cooked, boiled, drained, without salt	11019	24

Continued

Table 5.2 (Continued)

Description	NDB no.	mg 100 g^{-1}	Description	NDB no.	mg 100 g^{-1}
Kohlrabi, cooked, boiled, drained, without salt	11242	54	Grape juice, frozen concentrate, sweetened, diluted with three volume water, with added vitamin C	09137	24
Lemons, raw, without peel	09150	53	Peas, edible-podded, frozen, cooked, boiled, drained, without salt	11303	22
Orange juice, raw	09206	50	Tomato products, canned, paste, without salt added	11546	22
Peas, edible-podded, boiled, drained, without salt	11301	48	Turnip greens, frozen, cooked, boiled, drained, without salt	11575	28
Cauliflower, raw	11135	46	Rutabagas, cooked, boiled, drained, without salt	11436	19
Brussel sprouts, frozen, cooked, boiled, drained, without salt	11101	46	Collards, cooked, boiled, drained, without salt	11162	18
Cranberry juice cocktail, bottled	14242	42	Sauerkraut, canned, solids and liquids	11439	15

Source: Data from United States Department of Agriculture, Agricultural Research Service, 2006, USDA Nutrient Database for Standard Reference, Release 19, Nutrient Data Laboratory Home Page, http://www.nal.usda.gov/fnic/foodcomp, Riverdale, MD, Nutrient Data Laboratory, USDA.

Table 5.3 Dietary Reference Intakes and Tolerable Upper Intake Levels for Vitamin C

Life stage	DRI (mg d^{-1})	UL (mg d^{-1})
Infants (months)		
0–6	40	[a]
7–12	50	[a]
Children (years)		
1–3	**15**	400
4–8	**25**	650
Males (years)		
9–13	**45**	1200
14–18	**75**	1800
19–30	**90**	2000
31–50	**90**	2000
51–70	**90**	2000
>70	**90**	2000
Females (years)		
9–13	**45**	1200
14–18	**65**	1800
19–30	**75**	2000
31–50	**75**	2000
51–70	**75**	2000
>70	**75**	2000
Pregnancy (years)		
≤18	**80**	1800
19–30	**85**	2000
31–50	**85**	2000
Lactation (years)		
≤18	**115**	1800
19–30	**120**	2000
31–50	**120**	2000

Bold type: Recommended dietary allowance; ordinary type: adequate intake (AI).

[a] Not possible to establish; source of intake should be formula and food only.

Source: Food and Nutrition board, Institute of Medicine, *Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Corotenoids*, National Academy of Sciences Press, Washington, DC, 2000, Chap. 5

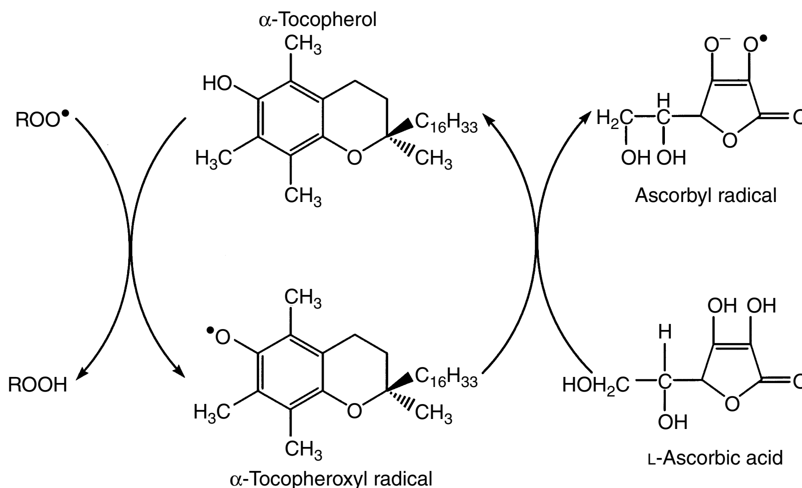


Figure 5.1 Regeneration of α-tocopherol from the α-tocopheroxyl radical by ascorbic acid. (Modified from Buettner, G. R., The pecking order of free radicals and antioxidants: lipid peroxidation, α-tocopherol, and ascorbate, *Arch. Biochem. Biophys.*, 300, 535, 1993; Eitenmiller, R. and Lee, J., *Vitamin E: Food Chemistry, Composition and Analysis*, Marcel Dekker, Inc., New York, 2004, Chap. 3.)

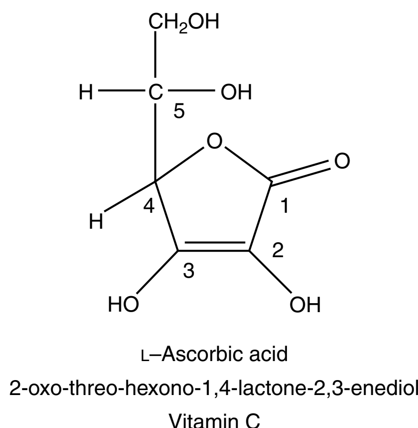


Figure 5.2 Structure of L-ascorbic acid.

Alignment of ascorbic acid in the aqueous phase near the tocopheroxyl radical in the micelle is necessary for regeneration. The ascorbyl radical is recycled by various enzyme systems in the aqueous phase. Other roles include participation in immune response, iron absorption, and protection of keratinocytes through modulation of gene expression as a result of oxidative stress.[18–20]

5.2 Properties

5.2.1 Chemistry

5.2.1.1 General properties

L-Ascorbic acid ($C_6H_8O_6$) is the trivial name for vitamin C, which is the accepted International Union of Pure and Applied Chemistry–International Union of Biochemistry (IUPAC–IUB) Commission on Biochemical Nomenclature name. The systematic chemical designation is 2,3-endiol-L-gulonic acid-γ-lactone.[21] The structure is given in Figure 5.2. L-Ascorbic acid is the United States Pharmacopeial Convention (USP) standard. Vitamin C refers to compounds exhibiting full or partial biological activity of L-ascorbic acid. These include esters of ascorbic acid such as ascorbyl palmitate, 100% relative activity, synthetic forms such as 6-deoxy-L-ascorbic acid with 33% relative activity and the primary oxidized form of L-ascorbic acid. L-Ascorbic acid and L-dehydroascorbic acid are the primary dietary sources of vitamin C.[7] L-Dehydroascorbic acid was considered to have as high as 80% bioequivalency to L-ascorbic acid;[22] however, recent research has indicated that it can have as low as 10% of the activity of L-ascorbic acid when measured with inherently scorbutic rats.[23] If this finding relates to the biological activity of L-dehydroascorbic acid in humans, many currently available food composition values that combine L-ascorbic acid and L-dehydroascorbic acid to present a value for total ascorbic acid would need to be revised to give a clear picture of the active amount of ascorbic acid in specific foods. Since routine processing and storage can lead to conversion of L-ascorbic acid to L-dehydroascorbic acid in significant amounts, analytical methods that rely on oxidation of L-ascorbic acid to L-dehydroascorbic acid to give a measure of total L-ascorbic acid would become obsolete. Also, more emphasis would be placed on chromatographic methods capable of resolving the various oxidation products from L-ascorbic acid. The finding also places doubt on the concept that all animals effectively use L-dehydroascorbic acid and that the only meaningful measure for biological active forms is the measure that sums the L-ascorbic acid and L-dehydroascorbic acid.[24]

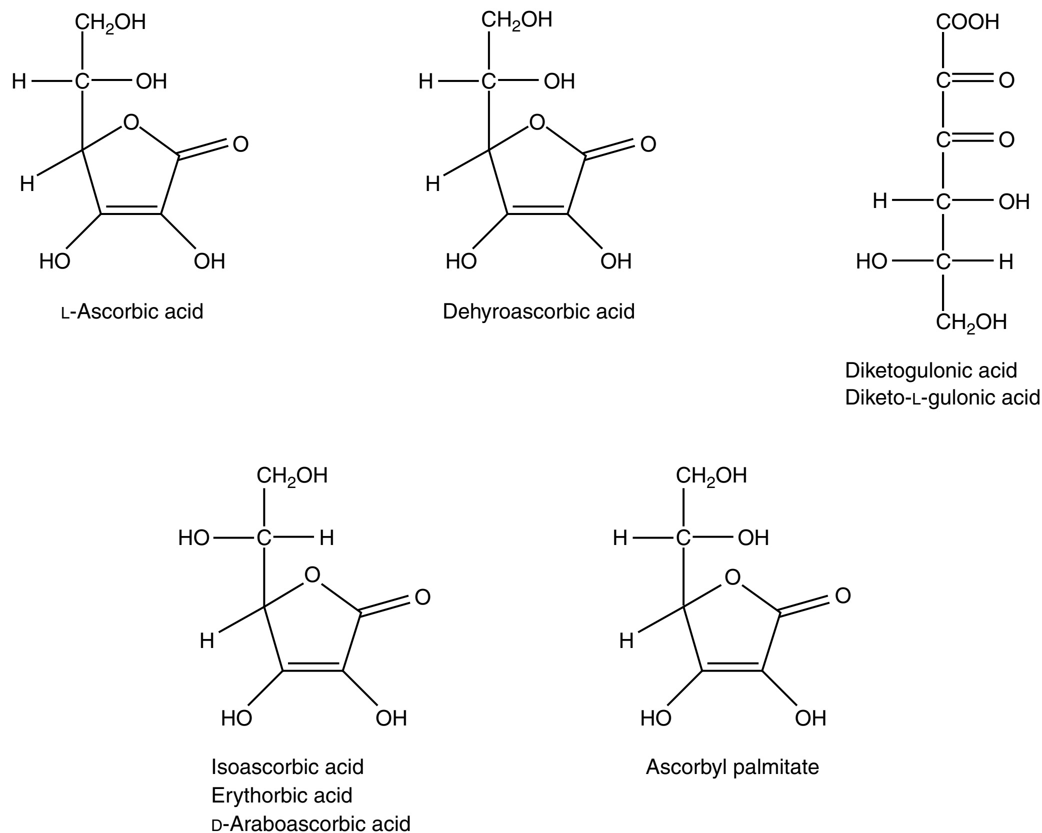


Figure 5.3 Structures of L-ascorbic acid and related compounds.

L-Ascorbic acid has chiral centers at carbons 4 and 5 and can exist in four stereoisomeric forms. Enantiomeric pairs are L- and D-ascorbic acid and L- and D-araboascorbic acid. L-Ascorbic acid and D-araboascorbic acid (more commonly known as D-isoascorbic acid) or erythorbic acid, are epimers differing in orientation of the hydrogen and hydroxyl on carbon 5.[25] We will use the term isoascorbic acid throughout this chapter. Structures of various ascorbic acid forms are given in Figure 5.3. Isoascorbic acid is not present in foods but is synthesized commercially for its antioxidant properties and commonly used in cured meat products to prevent oxidation and protect color. The stereoisomers have no biological activity other than a small amount from isoascorbic acid (2.5%–5% relative to L-ascorbic acid).[10,26] The use of isoascorbic acid as an antioxidant in foods, usually processed meats, and its low biological activity led to the development of liquid chromatography (LC) methods capable of quantitating the individual isomers. Most other assay techniques will not differentiate the epimers. Because isoascorbic acid is used in some processed foods, erroneously high vitamin C values will be found if improper methodology is used.

Physical properties of L-ascorbic acid, its salts, and ascorbyl palmitate are given in Table 5.4. L-Ascorbic acid is a white to slightly yellow crystalline powder with high water solubility (30 g 100 mL^{-1}) at ambient temperature. Its salts have higher water solubility. All commercial forms except fatty acid esters such as ascorbyl palmitate are insoluble in fats and oils. L-Ascorbic acid is a widely used food additive with many functional roles, many of which are based upon its oxidation–reduction properties. Its functional roles include its uses as a nutritional food additive, antioxidant, browning inhibitor, reducing agent, flavor

Table 5.4 Physical Properties of L-Ascorbic Acid and Related Compounds

Substance[a]	Molar mass	Formula	Solubility	Melting point °C	Crystal form	Absorbance[b]			
						λ max nm	$E^{1\%}_{1\,cm}$	ε × 10^{-3}	Solvent
Ascorbic acid CAS No. 50-81-7 **867**	176.13	$C_6H_8O_6$	Sol in water 30 g 100 mL^{-1}	190–192 (dec.)	Monoclinic platelets and needles	245 265	695 940	[12.2] [16.6]	Water, pH 2.0 Water, pH 6.4
			Sl sol in alcohol		White or yellow				
			Insol in ether, $CHCl_3$, benzene, petroleum ether, oils, and fats						
Na ascorbate CAS No. 134-03-2 **8723**	198.11	$C_6H_7O_6Na$	Sol in water 90 g 100 g^{-1}	—	White to sl yellow powder	—	—	—	—
Ca ascorbate CAS No. 5743-27-1 **1688**	390.31	$(C_6H_7O_6)_2Ca$	Sol in water 5 g 100 g^{-1} Sl sol in alcohol Insol in ether	—	White to sl yellow crystalline powder				
Ascorbyl palmitate	414.54	$C_{22}H_{38}O_7$	Sl sol in oils Sol in alcohol 22 g 100 mL^{-1}	—	White to sl yellow powder	—	—	—	—

[a] Common or generic name; CAS No.: Chemical abstract service number, bold print designates the Merck Index monograph number.
[b] Values in brackets are calculated from corresponding $E^{1\%}_{1\,cm}$ values.

Sources: Budavari, S., *The Merck Index*, 13th ed., Merck and Company, Whitehouse Station, NJ, 2001, p. 139; Friedrich, W., *Vitamins*, Walter de Gruyter, Berlin, 1998, Chap. 14; Committee on Food Chemicals codex, *Food Chemicals Codex*, 5th ed., National Academy of Sciences, Washington, DC, 2004, p. 36–37.

stabilizer, modifier and enhancer, color stabilizer, dough modifier, and in many other capacities. Ascorbyl palmitate was developed to provide an ascorbic acid form with greater lipid solubility for use in antioxidant preparations. Ascorbyl palmitate is highly effective synergistically with primary antioxidants including phenols and tocopherols.

5.2.1.2 Spectral properties

Absorption properties are dependent upon the ionic species present and, therefore, dependent upon the pH of the aqueous media. Ball[25] provides an excellent review of the spectral properties of L-ascorbic acid. E1% 1 cm values for L-ascorbic acid are 695 at pH 2.0 and 940 at pH 6.0 (Table 5.4). Above pH 5.0, L-ascorbic acid exists predominantly as the monoanion and has maximal absorption at 265 nm. Undissociated, at more acid pH levels, maximal absorption occurs at 244–245 nm. Fully dissociated, above pH 12.0, maximal absorption occurs at 300 nm.

L-Ascorbic acid does not fluoresce; however, derivatization with *o*-phenylenediamine to form a highly fluorescent product is used advantageously in chemical and LC methods discussed in Section 5.3.

5.2.2 Stability

Crystalline L-ascorbic acid is highly stable in the presence of oxygen when water activity remains low.[24] In solution, the strong reducing properties of the vitamin can lead to rapid and excessive oxidative changes with conversion to dehydroascorbic acid. Irreversible hydrolysis of L-dehydroascorbic acid produces the biologically inactive 2,3-diketo-L-gulonic acid. Reducing agents can convert the dehydro form back to L-ascorbic acid in biological systems. Enzymatic conversion of L-dehydroascorbate to L-ascorbic acid by glutathione dehydrogenase is an important biological defense against oxidative stress. Oxygen, temperature, light, metal catalysts, pH, and the possible presence of ascorbic acid oxidase in biological systems interact to produce a complex set of interactions influencing oxidative stability. Metal catalysts can increase degradation rates compared to uncatalyzed oxidation. Rates increase as pH increases through pK_1 (4.04).[30]

L-Ascorbic acid can oxidize through one- or two-electron transfers. One-electron reductions utilize the transition through the L-ascorbic acid free radical (semidehydroascorbic acid or monodehydroascorbic acid). At physiological pH, a bicyclic radical is formed with the loss of a proton. The anion radical is the intermediate in the reversible redox system formed by L-ascorbic acid and L-dehydroascorbic acid. Reducing agents and glutathione dehydrogenase convert L-dehydroascorbic acid back to L-ascorbic acid, completing the oxidation-reduction cycle. Classic free-radical termination occurs by reduction of a free radical with L-ascorbate. An electron is transferred to the free radical from ascorbate, producing an ascorbate radical, which acts as a redox agent. The ascorbate radical interacts with itself, forming a 1:1 mixture of L-ascorbic acid and dehydroascorbic acid.[31]

Two-electron reductions occur when transition metals catalyze L-ascorbic acid oxidation. A ternary complex forms between the metal, L-ascorbic acid and oxygen, and two p electrons shift from L-ascorbic acid to oxygen through the transition metal.[32] The complex then dissociates with the formation of L-dehydroascorbic acid, hydrogen peroxide and metal ion. Unless converted back to L-ascorbic acid, L-dehydroascorbic acid can be quickly hydrolyzed to biologically inactive 2,3-diketo-L-gulonic acid (Figure 5.3).

In foods, pH greatly influences oxidative stability. At low pH levels, the fully protonated form is quite stable. Maximal stability usually occurs between pH 4 and 6;[33] however, degradation rates are dependent on oxygen availability, the presence of antioxidants, thermal processing conditions, transition metal catalysis, oxidizing lipid effects, presence of reducing substances, the presence of ascorbic acid oxidase, and the multitude of possible

interactions. Cooking losses depend upon degree of heating, leaching into the cooking medium, surface area exposed to water and oxygen, pH, presence of transition metals, and any other factors that facilitate the oxidation of L-ascorbic acid and its conversion into nonbiologically active forms. L-Ascorbic acid is a characteristic reductone and enters into the nonenzymatic Maillard browning reaction. Browning in a food product can significantly reduce vitamin C content.

The quite extreme lability of L-ascorbic acid at or near physiological pH is a primary consideration in all analytical procedures. Specific extraction procedures designed to stabilize the vitamin are discussed in Section 5.3.

5.3 Methods

Vitamin C is the only water-soluble vitamin not assayed microbiologically. Rapid advances were made after the guinea pig bioassay was developed in 1922 to measure antiscorbutic activity.[34] Methodology has advanced from the bioassay to instrumentally advanced spectrophotometric, fluorometric, electrochemical, and chemiluminescence methods. Chromatographic procedures, primarily liquid chromatography, and capillary electrophoresis provide excellent means to resolve L-ascorbic acid, L-dehydroascorbic acid, and D-isoascorbic acid. These separation techniques used with ultraviolet/visible (UV/visible), fluorescence, or electrochemical detectors provide selective and sensitive means to quantify L-ascorbic acid and its isomers from complex biological matrices. Liquid chromatography coupled to mass spectrometry (LC-MS) has been used less frequently for vitamin C analysis compared to its use in other water-soluble vitamin studies.

Several excellent reviews covering the many different aspects of vitamin C analysis are available.[24,25,35–43] Pachla et al.[36] in 1985, classified vitamin C methods into spectroscopic, electrochemical, enzymatic, and chromatographic techniques. Capillary electrophoretic methods must now be added to this classification. In addition, chemiluminescence methods have been successfully applied to vitamin C analysis since the Pachla et al. review.[36]

Published regulatory and handbook methods are summarized in Table 5.5.[44–52,53–55] Several of these procedures are discussed in Section 5.3.2, since they represent the classical approaches to vitamin C analysis used in laboratories worldwide. The basic chemistry of these procedures forms the basis of newer, more instrumentally advanced methods.

5.3.1 Extraction procedures

Owing to the labile nature of vitamin C, extraction procedures are designed to stabilize the vitamin. Cooke and Moxon[38] reviewed the literature up to 1981 and found that 20 or more extraction solutions were used by various researchers working with a large number of biological matrices. Extraction solutions should maintain an acidic environment, chelate metals, inactivate ascorbic acid oxidase, limit soluble oxygen, and precipitate starch and proteins. Choice of the extraction solution depends upon the sample matrix and the determinative procedure. Extractants that usually limit L-ascorbic acid destruction to less than 5% include 3%–6% metaphosphoric acid containing acetic or sulfuric acid or 0.005 M ethylenediaminetetraacetic acid (EDTA).[38] Metaphosphoric acid, while not compatible to some LC procedures, has been the most commonly used extractant. Metaphosphoric acid inhibits L-ascorbic acid oxidase, inhibits metal catalysis, and precipitates proteins that aid in extract clarification.[35] Starch is problematic in that it interferes with colorimetric titrations and fluorometric assays. Addition of ethanol or acetone to the metaphosphoric extract precipitates solubilized starch.[56,57] This step is necessary for analysis of many vegetables including potatoes, legumes, and corn by spectroscopic methods. Acetone is also useful to remove metabisulfite and sulfur dioxide from dehydrated fruit products and fruit juices. These

Table 5.5 Regulatory and Handbook Methods of Analysis for Ascorbic Acid

Source	From	Methods and application	Approach	Most current cross reference
***U.S. Pharmacopeia, National Formulary*, 2006, USP 29/NF 24 Nutritional Supplements Official Monographs**[44]				
1. Pages 2492, 2391, 2395, 2398, 2413, 2394	Ascorbic Acid, vitamin capsules/tablets w/wo minerals, oral solution	Ascorbic acid in oil– and water-soluble	Titration Dichlorophenolindolphenol or automated fluorescence Ex λ = 335 Em λ = 426	None
2. Pages 2492, 2426, 2429, 2431, 2436	Ascorbic acid, capsules/ tablets w/wo minerals	Ascorbic acid in water-soluble vitamin	Titration Dichlorophenolindol phenol or automated fluorescence Ex λ = 335 Em λ = 426	None
3. Page 194	Ascorbic acid	Ascorbic acid (NLT 99.0%, NMT 100.5%)	Titration, iodine	None
4. Pages 194–195	Ascorbic acid	Ascorbic acid injection, oral solution, tablets	Titration, dichlorophenolindol phenol	None
5. Page 345	Calcium ascorbate	Calcium ascorbate	Titration, iodine	None
***British Pharmacopoeia*, 2007**[45]				
1. Pages 178–179	Ascorbic acid	Ascorbic acid	Titration	None
2. Page 2328	Ascorbic acid	Ascorbic acid injection	Titration	None
3. Pages 2328–2329	Ascorbic acid	Ascorbic acid tablets	Titration	None
4. Page 179	Ascorbyl palmitate	Ascorbyl palmitate	Titration	None
5. Pages 2987–2989	Ascorbic acid	Pediatric vitamins A, C, and D oral drops	Titration	None
6. Pages 2989–2990	Ascorbic acid	Vitamins B and C injection	Titration	None

Continued

Table 5.5 (Continued)

Source	From	Methods and application	Approach	Most current cross reference
***AOAC Official Methods of Analysis*, 18th ed., 2005**[46]				
1. 5.1.14	Ascorbic acid	AOAC Official Method 967.21 vitamin C in juices and vitamin preparations	Titration, dichlorophenolindolphenol	*J. Assoc. Off. Anal. Chem.*, 50, 798, 1967[47]
2. 45.1.15	Ascorbic acid	AOAC Official Method 967.22 vitamin C in vitamin preparations	Fluorescence Ex λ = 350 Em λ = 430	*J. Assoc. Off. Anal. Chem.*, 48, 1248, 1965;[48] 50, 798, 1967[47]
3. 45.1.16	Ascorbic acid	AOAC Official Method 984.26 vitamin C in foods	Semiautomated Fluorescence Ex λ = 365 Em λ = 440	*J. Assoc. Off. Anal. Chem.*, 66, 1371, 1983[49]
4. 50.1.09	Ascorbic acid	AOAC Official Method 985.33 vitamin C in Ready-To-Feed Milk-Based Infant Formula	Titration, dichlorophenolindol phenol	*J. Assoc. Off. Anal. Chem.*, 68, 514, 1985[50]
***European Committee for Standardization*, 2003**[53]				
1. EN14130	Ascorbic acid	Foodstuffs—determination of vitamin C by HPLC	LC 265 nm	*J. Agric. Food Chem.*, 29, 927, 1981[54] *Ann. Fais. Exp. Chem.*, 90, 217, 1997[55]
***American Association of Cereal Chemists, Approved Methods*, vol. 2, 2000**[51]				
AACC 86-10	Ascorbic acid Ascorbic acid in cereal products	Ascorbic acid in cereal products	Spectrophotometric 500 nm	*AOAC International*, 18th ed., 2005[46]
***Food Chemicals Codex, 5th ed.*, 2004**[52]				
1. Page 36	Ascorbic acid	Ascorbic acid (NLT 99.0%, NMT 100.5%)	Titration, iodine	None
2. Page 36	Ascorbyl palmitate	Ascorbyl palmitate (NLT 95.0%)	Titration, iodine	None

reducing agents interfere with the 2,6-dichloroindophenol (DCIP) titration based on reduction of the oxidized dye. EDTA is active as a chelator in vitamin C extractants. It is effective for copper chelation in metaphosphoric acid and trichloroacetic acid, but ineffective for oxalic acid chelation.

All extraction procedures should be completed rapidly in subdued light to limit light-catalyzed oxidative reactions. Particle size reduction methods should avoid heat build up. Whenever possible, the sample should be overlayered with nitrogen during initial sample compositing procedures.[24] Freeze-drying is not recommended for sample concentration or preservation since vitamin C stability decreases in the porous matrix.[38] When high moisture samples are blended, the stabilizing extractant should be added before blending.

Stability of total ascorbic acid (L-ascorbic acid + L-dehydroascorbic acid) in serum can be extended for long periods under proper conditions. Addition of dithiothreitol or metaphosphoric acid (50 g L^{-1}) effectively stabilizes the vitamin when the serum is held frozen at –70°C.[58,59] The National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 970, Ascorbic Acid in Serum, is stabilized with metaphosphoric acid at 50 g L^{-1}.[60] Stability in the blood from the time it is drawn to the time the plasma is prepared, stabilized, and analyzed is problematic. Terzuoli et al[61] recommended a lithium heparin treatment with samples kept at room temperature for transport of no more than 2 h from the time the blood is drawn for maximal L-ascorbic acid stability. Ching et al.[62] recommended dipotassium EDTA over lithium hepararin treatment with separation within 2 h to minimize degradation. Margolis and Park[63] showed that degradation can occur upon placement of L-ascorbic acid solutions in autosampler vials before analysis. They showed that the inside surface of the glassware can contain materials such as trace amounts of metals that can rapidly degrade L-ascorbic acid. The authors stated that the sample tubes for collection, processing, and storage are also subject to this problem. They evaluated nine lots of vials from five suppliers and found high variation in the stability of L-ascorbic acid stored in the different vials. Effective elimination of the degradation involved soaking the vials in 0.5 mol L^{-1} NaOH for 30 min; rinsing with distilled, deionized water; soaking in 1 mol L^{-1} HCl; and again rinsing with distilled, deionized water.

Sodium thiosulfate (0.04% w/v) was an effective stabilizer for L-ascorbic acid dissolved in distilled water before UV analysis.[64] It was more effective than either sodium metabisulphite and sodium sulphite. Rates of decomposition within 60 min after dissolution were sodium thiosulphate (0.04%) < sodium metabisulphite (0.4%)< sodium sulphite (0.16%).

Rizzolo et al[65] showed that handling and storage of fresh fruit was critical to stability of L-ascorbic acid before analysis. These authors sampled pears by avoidance of extensive cutting to minimize induction of ascorbate oxidase and other oxidases that catalyze L-ascorbic acid oxidation, freezing each sample in liquid nitrogen and storing samples before extraction at –80°C. Hernãndez et al.[66] compared 3% metaphosphoric acid–8% acetic acid to 0.1 oxalic acid for extraction of ascorbic acid from tropical fruits. The metaphosphoric–acetic extractant was suitable for all fruits studied; whereas, oxalic acid gave variable results.

5.3.2 Classical approaches to vitamin C analysis

5.3.2.1 Oxidation–reduction methods

5.3.2.1.1 2,6-Dichloroindophenol titration. The DCIP titration was introduced by Tillmans in 1930.[67] DCIP is reduced by L-ascorbic acid to a colorless solution from the deep blue color of the oxidized dye (Figure 5.4). L-Ascorbic acid is oxidized to dehydroascorbic acid and excess dye remains pink in acid solution, forming the visual endpoint of the titration. Absorbance at 518 nm can be used alternatively to visual end point determination.

Several important deficiencies exist with the method. Most importantly, the titration is limited to quantitation of L-ascorbic acid. Dehydroascorbic acid will not be measured unless

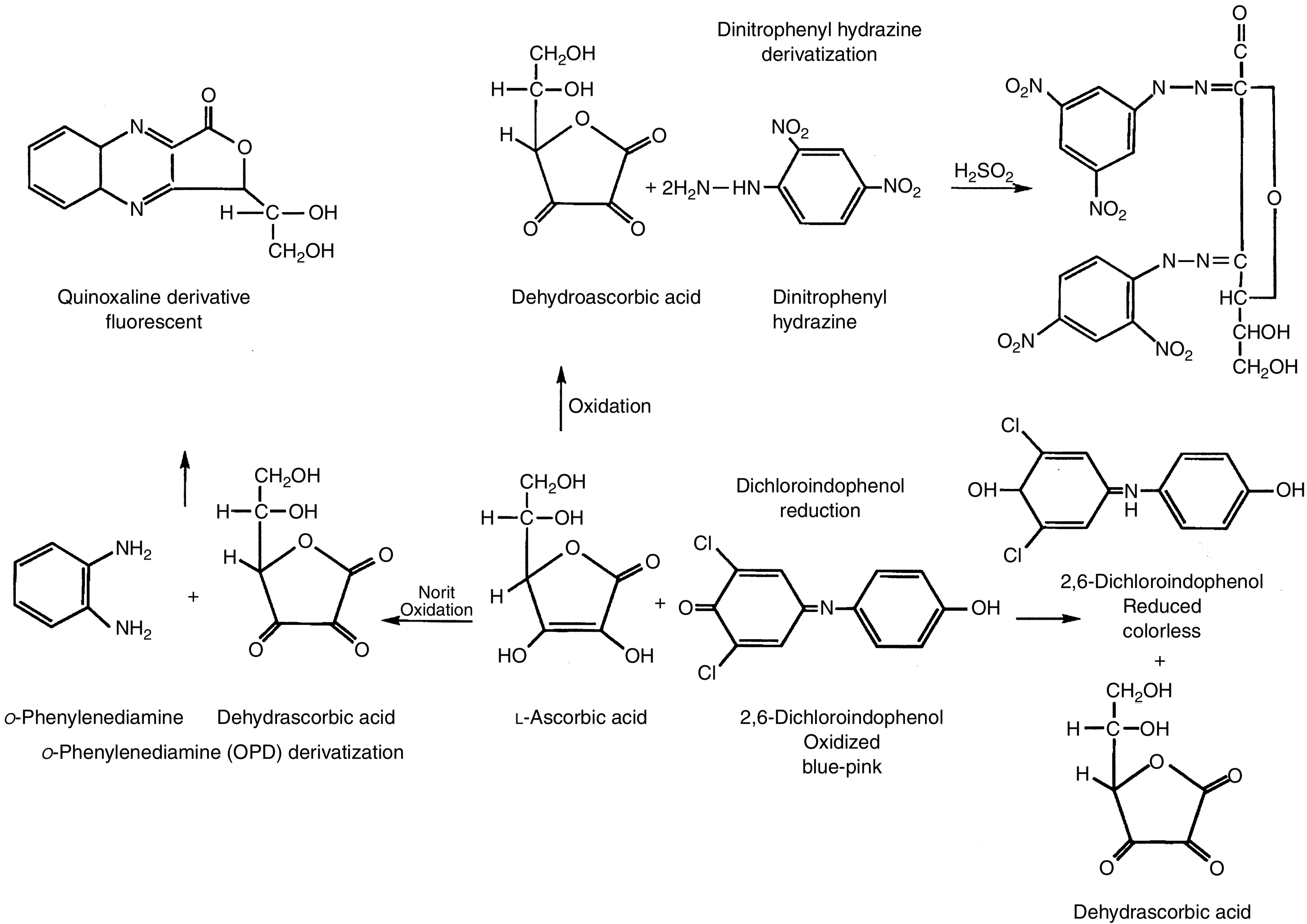


Figure 5.4 L-Ascorbic acid reactions important to analysis of vitamin C. (Adapted from Eitenmiller, R. R. and Landen, W. O., Jr., Vitamins, In *Analyzing Food for Nutrition Labeling and Hazardous Contaminants*, Jeon, I. J. and Ikins, W. G., eds., Marcel Dekker, New York, 1995, chap. 9. With permission.)

it is reduced to ascorbic acid. The titration will not distinguish between L-ascorbic acid and isoascorbic acid. The method cannot be used for vitamin C analysis of processed and cured meats containing isoascorbic acid. DCIP titration can be used for fresh juices and multivitamins that do not contain excessive amounts of copper or iron.[35] For processed or cooked foods known to contain copper, iron, or tin, other methods capable of measuring dehydroascorbic acid in addition to L-ascorbic acid should be used to quantitate total vitamin C.[24] Highly colored extracts from fruit and vegetables can mask the color change at the titration's end point.

Reduction of DCIP is not limited to L-ascorbic acid, and any reducing substance present in the sample can reduce the dye. Such interferences can lead to erroneously high measurements, if not recognized. Substances that can interfere include cuprous, ferrous, and stannous ions, sulfite, thiosulfate, tannins, betanin, cysteine, glutathione, and reductones generated by nonenzymatic browning. Several method modifications have been introduced to eliminate or minimize the effects of interferences on the DCIP titration.[35,36,38]

A solid-phase extraction (SPE) procedure was developed that expands the DCIP titration to highly colored multivitamins, soft drinks, and fruit and vegetables.[68] Further, the cleanup step removes copper, iron, sulfite, and other interfering reducing substances, such as cysteine and glutathione. C_{18} silica impregnated with 2,29-bipyridyl-2,9-dimethyl-1,10-phenathroline (neocuproine) and *N*-ethylmaleimide removes Fe(II) and Cu(I) and sulfhydryl compounds, respectively. The method provides for determination of L-ascorbic acid and L-dehydroascorbic acid by reducing the L-dehydroascorbic acid back to L-ascorbic acid with cysteine before the SPE step. This procedure is simple and increases the sensitivity of the DCIP procedure. Incorporation of the SPE step into existing regulatory methods could decrease problems associated with existing titration procedures.

AOAC Official Method 967.21,[46] Ascorbic Acid in Vitamin Preparations and Juices, 2,6-Dichloroindophenol Titrimetric Method, AOAC Official Methods of Analysis 45.1.14—AOAC Method 967.21 was recommended for the analysis of L-ascorbic acid in beverages and juices for nutritional labeling purposes.[69] However, the method is routinely applied worldwide to other food matrices because of its simplicity. If the deficiencies of the method are recognized by the analyst, the procedure provides reliable measures for L-ascorbic acid provided that the food does not contain appreciable quantities of reducing substances and L-dehydroascorbic acid.[24] Application of the SPE cleanup discussed previously could greatly expand the use of the DCIP titration since many laboratories still must rely on simple, noninstrumental approaches to food analysis.

The method includes the following steps:

1. *Extraction*: L-Ascorbic acid is extracted from dry materials with metaphosphoric acid containing glacial acetic acid. The solution is stable for 7 to 10 days when refrigerated. The extractant is used to dilute juices or other liquid samples. For analysis of highly basic materials, the extractant is prepared by replacing water in the extractant with 0.3 N H_2SO_4. When whole food products are extracted, the food should be blended in the metaphosphoric acid extractant. Following filtration or centrifugation, the residue should be extracted at least one additional time by blending in the extractant. Proper extraction including light protection, speed, and quantitative techniques result in recoveries greater than 95% with minimal formation of dehydroascorbic acid.[24]
2. *Titration*: Clarified extracts are titrated with standard dye prepared by dissolving 50 mg DCIP Na salt (Eastman Kodak No. 3463) in 50 mL water containing 42 mg $NaHCO_3$, with dilution to 200 mL with water. The dye solution is filtered and stored under refrigeration in an amber glass container. The DCIP concentration is expressed as mg L-ascorbic acid equivalent per 1.0 mL dye solution. The equivalency factor is

determined by adding 2 mL of standard L-ascorbic acid (1.0 mg mL^{-1}) to 5 mL of the extractant and titrating rapidly with dye solution to a pink color that persists for 5 s. This should require approximately 15 mL of the DCIP solution. A blank determination is run by titrating 7 mL of the extractant containing water equal to the average volume of dye required to titrate the ascorbic acid standard. Blanks should approximate 0.1 mL DCIP solution, which provides an immediate check on reagent quality.

Notes

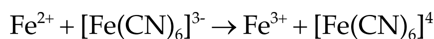
1. The AOAC International Methods of Analysis[46] provides specific tests to check for interfering levels of Cu (I), Fe (II), and Sn (II). Application of the SPE cleanup[68] procedure clearly will improve application of AOAC Method 967.21 to complex matrices encountered in processed food, beverages, medical foods, and multivitamins.
2. The AOAC Official Method 985.33, Vitamin C (Reduced Ascorbic Acid) in Ready-to-Feed Milk-Based Infant Formula) (Chapter 50.1.09) is based on the DCIP titration. The method varies from Method 967.21 at the extraction stage of the method. EDTA is added to the metaphosphoric acid–glacial acetic acid extractant to ensure the removal of iron and copper interferences. Iron and copper salts are components of commercial infant formulas and many supplements.

5.3.2.1.2 Metal ion reduction. L-Ascorbic acid in biological samples can be measured by redox reactions in which L-ascorbic acid is used to reduce metal ions to produce colored products. Pachla et al.[36] reviewed metal ion redox methods in detail. These methods rely upon the formation of a stable colored complex between the reduced ion and a chelating agent. Reduction of Fe(III) to Fe(II) by L-ascorbic acid is the most common reaction, although many metal ion redox reactions have been utilized for L-ascorbic acid analysis. A chelator is added, which complexes with the reduced metal. The reduced metal–chelator complex is then measured by spectroscopic methods. With Fe(II), the most common chelating agents are 2,29-dipyridine, 2,4,6-tripyridyl-5-triazine, and ferrozine.[36]

More recent approaches to metal redox applications for L-ascorbic acid have entailed flow injection and sequential injection techniques with spectrophotometric methods designed to quantitate L-ascorbic acid and L-dehydroascorbic acid. A simple flow injection technique developed for pharmaceutical products used Fe(III) and hexacyanoferrate(III) as the chromogenic complexing reagents to produce Prussian Blue.[70]

The reaction sequence is the following:

1. Oxidation of Fe(II)



$$Fe^{2+} + [Fe(CN)_6]^{3-} \rightarrow Fe^{3+} + [Fe(CN)_6]^{4}$$

2. Formation of hexacyanoferrate(II) ferric complex

$$4Fe^{3+} + 3[Fe(CN)_6]^{4-} \rightarrow Fe_4[Fe(CN)_6]_3 \text{ Prussian Blue}$$

L-Ascorbic acid reduces Fe(III) to Fe(II) resulting in the formation of a deep blue soluble complex. When excess hexacyanoferrate(III) is present, the formation of Prussian Blue is measured at 700 nm. This procedure is detailed in Section 5.4.

5.3.2.2 Derivatization methods

5.3.2.2.1 o-Phenylenediamine. The *o*-phenylenediamine (OPD) condensation reaction with L-dehydroascorbic acid (Figure 5.4) represents one of the most useful derivatization

reactions to quantitate total vitamin C. The condensation reaction produces a highly fluorescent quinoxaline product (Ex λ = 350, Em λ = 430). AOAC International Method 967.22 was developed by Deutsch and Weeks[48] for analysis of total vitamin C (L-ascorbic acid + dehydroascorbic acid). This assay improved the scope and specificity of vitamin C methods existing at the time.[24] A detailed discussion of the method is presented by Brubacher et al.[71]

AOAC Official Method 967.22, Vitamin C (Total) in Vitamin Preparations, Microfluorometric Method—The AOAC Task Force on Methods for Nutrition Labeling recommended that Method 967.22 be used for most food matrices.[69] The method includes the following steps:

1. *Extraction*: Samples are extracted with the metaphosphoric acid–glacial acetic acid extractant used with the DCIP assay (Method 967.21). A 100 mL aliquot of the extract is vigorously mixed with 2 g of acid-washed Norit. The Norit oxidizes L-ascorbic acid to L-dehydroascorbic acid. The application of Norit oxidation by Deutsch and Weeks, [48] while simple in approach, greatly improved the accuracy of the methods used to report total vitamin C content of the food supply. Conversion of L-ascorbic acid to L-dehydroascorbic acid facilitates total vitamin C assay by OPD condensation with formation of the fluorescent quinoxaline and removes potential interfering substances without tedious cleanup steps.
2. *Quinoxaline Fluorescent Derivative Formation*: The Norit-treated extract is filtered and 5 mL of the filtrate is added to a 100 mL volumetric flask containing 5 mL sodium acetate solution and 75 mL water. Contents are diluted to 100 mL and 2 mL aliquots are transferred to fluorescence reading tubes. Five milliliters of OPD solution is added to each tube. After mixing, the tubes are incubated for 35 min at room temperature in the dark. Fluorescence is determined at Ex λ = 364, Em λ = 440. Standard L-ascorbic acid solution (100 mg mL^{-1}) is carried through the procedure.
3. *Blank Correction*: Blank corrections greatly improve specificity of the assay by accounting for substances other than dehydroascorbic acid that are in the sample extract and reactive with OPD or those that possess native fluorescence that escape Norit treatment.[24] The blank is produced by adding boric acid–sodium acetate buffer to the Norit-treated sample and standard solutions. The boric acid complexes with L-dehydroascorbic acid and prevents condensation with OPD. The fluorescence in the boric acid blank corrects for nonspecific fluorescence in the sample extract.

Although few interferences occur in most biological samples, Deutsch and Weeks[48] summarized properties of potential interferences. These include the presence of α-keto groups that react with OPD, fluorescence properties of the L-dehydroascorbic acid quinoxaline derivative, and presence of adjacent *cis*-hydroxyl groups, which react with the boric acid in the blank. The assay is subject to quenching by impurities in the extract. Also, dehydroreductic acid, dehydroreductones, and alloxan interfere by producing fluorescence in the sample solution but not in the blank.[72] Dehydroreductones are formed by the Maillard reaction (nonenzymatic browning). Therefore, browned foods might be subject to error when assayed for total vitamin C by the procedure.[24] Physical interference results from the extraction of high-starch products such as potatoes, corn, beans, and processed, creamed vegetables.[56,57] The metaphosphoric acid extracts enough starch from these foods to interfere with the fluorescence measurement. Extracts containing starch show a characteristic opalescence, which is easily distinguished by trained analysts. The problem can be avoided by diluting the initial extract 1:1 with 95% ethanol to precipitate the starch. After removal of the starch, the assay is completed without further modifications. The L-ascorbic acid standard must be similarly treated.

The manual microfluorometric method was modified to semiautomated analysis by use of DCIP[73] and *N*-bromosuccinimide[74] oxidation in place of Norit oxidation and direct

addition of Norit slurry in metaphosphoric acid to the food sample to immediately oxidize L-ascorbic acid to L-dehydroascorbic acid during the extraction.[75] AOAC International Method 984.26, Vitamin C (Total) in Food, Semiautomated Fluorometric Method (Chapter 45.1.16)[46] was developed by Egberg et al.[75] These investigators added the Norit slurry directly to the metaphosphoric acid–food mixture, allowing adaptation of the method to a Technicon Autoanalyzer system. The semiautomated method gives data comparable to the manual method.[76,77] The modified method provides speed (40 assays per hour) and the same sensitivity and specificity as AOAC International Method 967.22. It has been recommended for possible application to a wide variety of food matrices.[25]

Robotics and flow injection analysis were combined to further automate the general procedure of AOAC Method 967.22.[78,79] Following extraction, the extract is passed through a C_{18} preparative column to remove interferences. The eluent is loaded into autosampler vials and assayed by flow injection analysis. Mercuric chloride is used to oxidize L-ascorbic acid to dehydroascorbic acid and then derivatized with OPD. Results of the automated method compare with results of the manual procedure.

5.3.2.2.2 2,4-Dinitrophenylhydrazine. 2,4-Dinitrophenylhydrazine (DNPH) reacts with ketone groups of dehydroascorbic acid under acidic conditions to form a red osazone derivative (Figure 5.4). The method was developed in 1943,[80] and several excellent reviews have been written on its application.[25,35,36] DNPH is useful for the analysis of total vitamin C if appreciable quantities of sugars are not present in the product. L-Ascorbic acid is oxidized to L-dehydroascorbic acid by Norit or DCIP. Derivatization is completed with the addition of DNPH and the color is produced upon acidification with sulfuric acid. Maximal absorbance occurs between 500 and 550 nm. Most methods measure the DNPH derivative at 520 nm. Specificity of the reaction for L-dehydroascorbic acid in complex matrices was attributed to the ability of DNPH to react faster with L-dehydroascorbic acid compared with other carbohydrates; color is produced more easily with DNPH derivatives of 5- and 6-carbon sugar-like compounds and through the ability to minimize formation of nonascorbic acid chromogens by carrying out the reaction at low temperatures.[36] Despite the specificity afforded by such factors, DNPH has not been used recently as extensively for food analysis as OPD derivatization. The methods do not compare in simplicity and specificity to the microfluorometric method for total vitamin C assay. DNPH methods for the differential determination of isoascorbic acid are available.[25] Pachla et al.[36] reviewed automated methods based upon DNPH for application to plasma and food analysis.

A rapid DNPH-based microtiter plate assay for ascorbic acid determination in plasma and leukocytes is available.[81] The microtiter plate method can be used to assay L-ascorbic acid in a leukocyte-rich fraction prepared from 1 mL of whole blood. The method is capable of high sample throughput, suitable for small sample volumes, and requires smaller amounts of reagents than traditional DNPH methods. Results are similar to standard spectrophotometric methods using DNPH. Because of the potential for use in many different laboratory situations, the microtiter plate assay is summarized in Section 5.4.

5.3.2.3 Enzymatic methods

Enzyme conversions of L-ascorbic acid to L-dehydroascorbic acid coupled to a determinative step such as direct spectrophotometric assay following decrease of L-ascorbic acid, OPD, other derivatization reactions, and electrochemical determination of oxygen uptake during the reaction have been used to assay L-ascorbic acid in biological samples. Ascorbate oxidase and ascorbate peroxidase activity represented by the following equations convert the L-ascorbic acid to the dehydro form.[25]

Ascorbate Oxidase

$$\text{L-Ascorbic acid} + {}^{1}\!/_{2}\, O_2 \rightarrow \text{L-Dehydroascorbic acid} + H_2O$$

Ascorbate Peroxidase

$$\text{L-Ascorbic acid} + H_2O_2 \rightarrow \text{L-Dehydroascorbic acid} + 2H_2O$$

A variety of enzyme sources have been used for the enzymatic conversion. An ascorbate oxidase spatula is available from Boehringer–Mannheim to convert L-ascorbic acid and isoascorbic acid to the dehydro forms before OPD derivatization and LC quantitation of the quinoxaline derivatives.[82] This procedure, summarized in Section 5.4, has been extensively used for quantitation of total vitamin C in foods.[83,84] Total vitamin C and isoascorbic acid can be quantitated at levels as low as 0.2 µg g^{-1}. L-Dehydroascorbic acid can be quantified by omitting the enzymatic oxidation. Ihara et al.[85] used ascorbate oxidase and OPD derivatization to develop a rapid automated method for the routine assay of L-ascorbic acid in serum and plasma. The assay had a sample throughput of 100 h^{-1}.

Ascorbate peroxidase oxidation of L-ascorbic acid to dehydroascorbic acid has been applied to the spectrophotometric assay of total vitamin C in foods. In more recently published methods, quiacol peroxidase from horseradish has been used.[86,87] This peroxidase is commercially available (Sigma Chemical Co.) and catalyzes the oxidation of L-ascorbic acid as well as quiacol. The direct spectrophotometric assay developed by Tsumura et al.[87] was tested on a wide variety of foods and no interferences were apparent. The method was more precise when compared to assays using DCIP and DNPH. The procedure is detailed in Section 5.4.

5.3.3 *Advances in the analysis of vitamin C*

5.3.3.1 *Spectroscopic and electrochemical detection combined with flow injection and sequential injection analysis*

Development of spectroscopic methods using spectrophotometric, fluorescence, and chemiluminescence detection and methods based on electrochemical detection are rapidly advancing. Some recent reviews include those by Arya et al.,[39,40] Zaporozhets and Krushshinskaya,[41] and Yebra-Biurrun.[42] Many of the spectrophotometric methods utilize the redox properties of L-ascorbic acid; thus, the reduction of Fe(III) to Fe(II) and Cu(II) to Cu(I) are frequently incorporated into newer methods. Metal ion reduction is usually coupled with the complex of the reduced metal with various dyes exhibiting color change upon complexation. Color changes, while easily measured spectrophotometrically, can be used for simple, visual tests or development of test strips for approximation of vitamin C content. Dye that have been used include 2,2′-dipyridyl, pyridine-2,6-dicarboxylic acid, *p*-carboxyphenyl fluorone, 4-(2-pyridylazo) resorcinol, 1,10-phenanthroline, 2,4,6-tri(2-pyridyl)-1,3, 5-trianzine, ferrozine, and many others.

It is very evident from recent literature that the most dramatic change in L-ascorbic acid analysis has been the combination of flow injection analysis (FIA) and more sophisticated sequential injection analysis (SIA) with proven approaches of vitamin C analysis to provide rapid analytical methods. The review paper by Yebra-Biurrun[42] showed the trend very clearly. The author summarized the advantages of FIA as follows:

1. High sample throughput
2. Low sample volume

3. Low consumption of reagents
4. High reproducibility
5. Simple automation
6. Low contamination risks
7. Selectivity through kinetic discrimination
8. Small bench space needs
9. Limited need for labware

Such methods have been used with spectrophotometric, fluorescence, chemiluminescence, and electrochemical-based determinations. Sequential injection analysis was first proposed by Ruzicka and Marshall[88] as a logical, next generation improvement in FIA introduced in 1975 by Ruzicka and Hansen.[89] As previously introduced, FIA systems include a two-channel pump, an injection valve, a coiled reactor, and a detector. The reagent is continuously added to the carrier stream, which allows the injected sample to be merged with the reagent. A concentration gradient forms in the sample zone from the reagent–analyte reaction, which can be detected by almost any detection mode. In SIA, the following events as outlined by Ruzicka[90] occur

1. The measuring cycle begins by aspiration of a precise volume of sample solution through a multiposition valve into a holding coil by the pump moving in reverse.
2. The valve is switched to the detector position and the pump propels the sequenced zones forward.
3. The cores of the sequenced zones penetrate each other as the central fluid flow moves at a rate twice the speed of the mean flow with fluid near the walls moving at slower rates.
4. Radical mixing is promoted by coil geometry, and the zones mix with formation of the reagent–analyte product.
5. The reactant zone can be continuously pumped through the detector or stopped (stopped-flow) within the detector for reaction kinetic studies.

The engineering of the system allows unlimited designs with advanced computer control. Diagrams of FIA and SIA systems are given in Figure 5.5. Brief description of spectroscopic methods (spectrophotometric, fluorometric, and chemiluminescence) are provided in Table 5.6. [91–132] For methods using FIA or SIA, spectrophotometric and electrochemical (EC) detection are most commonly used.[42] More recent methods based on EC are presented in Table 5.7.[133–157]

5.3.3.2 Capillary electrophoresis

Capillary electrophoresis (CE) has been extensively used for analysis of fat- and water-soluble vitamins. The methodology is widely used for assay of vitamin C in pharmaceuticals and fruit and vegetable products. Heiger[158] reviewed CE principles, instrumentation, and modes of operation. Since its introduction, two basic techniques have been most used for vitamin C analysis, capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC). Each technique is highly versatile, faster, more efficient and cost-effective compared to more traditional methods.[159] A disadvantage is lower sensitivity compared to LC. However, for higher concentration matrices, supplements and fruit and vegetables, CE procedures are highly useful. Further, CE is very amenable to multianalyte assays (Chapter 14). CZE is normally applied to water-soluble analytes and is subject to column fouling by macromolecular components. This is proven to be a challenge for food analysis application. MECC was developed to overcome such problems and can be used to resolve neutral analytes on the basis of partitioning between the aqueous electrolyte and a

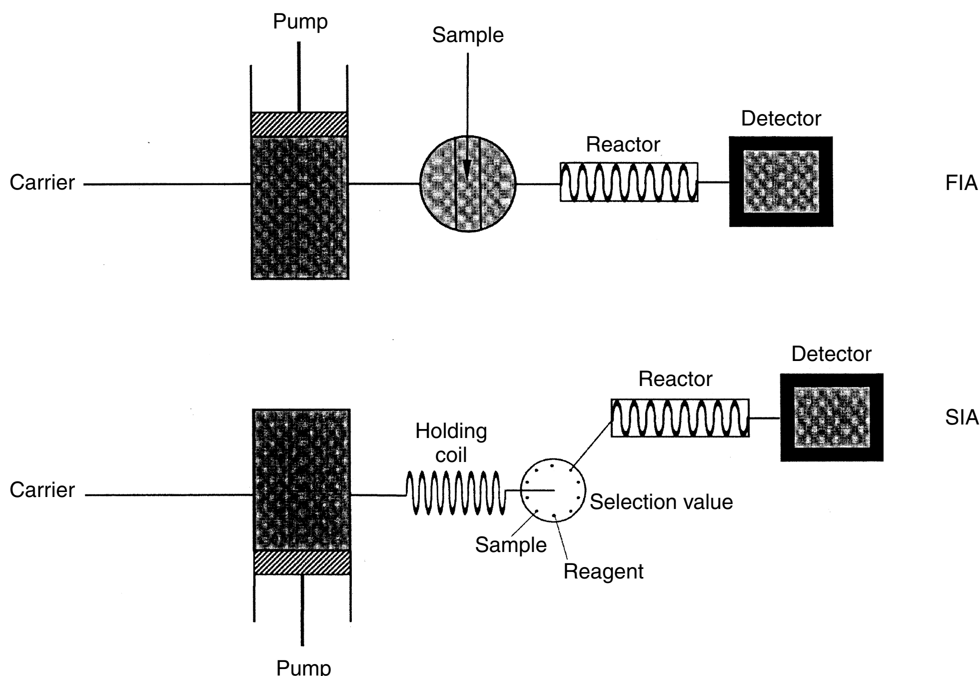


Figure 5.5 Schematic of a flow injection analysis (FIA) system and a sequential injection analysis (SIA) system.

pseudostationary phase of charged molecules.[160,161] Therefore, MECC is more adaptable to complex food matrices. However, hydrophobic analytes, such as fat-soluble vitamins, can precipitate out during electrophoresis owing to low solubility in aqueous MECC buffers. Microemulsion electrokinetic capillary chromatography (MEECC) was deveoped to overcome deficiencies of MECC with hydrophobic analytes.[161] For vitamin C, CZE and MECC techniques are widely used for pharmaceutical analysis. Some published methods are summarized in Table 5.8.[162–194]

5.3.3.3 Liquid chromatography

Liquid chromatography methods have been applied to pharmaceuticals and many types of biological samples for analysis of L-ascorbic acid and related compounds. The need to simultaneously assay L-ascorbic acid, L-dehydroascorbic acid, and isoascorbic acid from food has led to the development of excellent procedures for accurate assay of total vitamin C in the presence of isoascorbic acid.[25,43,195] Table 5.9 summarizes recent literature and some old method development publications of historical significance.[78,79,82–84,196–226,66,227–229] These methods were selected from quite a large pool of published papers and are not meant to be inclusive.

5.3.3.3.1 Extraction procedures for the analysis of vitamin C by LC. Most extraction procedures used for biological samples in conjunction with spectrophotometric oxidation–reduction based methods or derivatization procedures are compatible with resolution and detection modes used for LC analysis of vitamin C. The analyst must determine extractant compatibility to all components of the LC system. Summaries of recent methodology approaches provided in Table 5.9 show that metaphosphoric acid, mixtures of metaphosphoric acid with glacial acetic acid, trichloroacetic acid, citric acid, mixtures of citric acid

Table 5.6 Selected Spectroscopic Methods for Analysis of Vitamin C—Spectrophotometric, Fluorescence, and Chemiluminescence

Matrix	Description	Accuracy/precision	References
Spectrophotometric			
Fruits and Vegetables	Third order derivative UV spectrophotometry Extrema of 259.4 nm and 276.2 nm or 227 nm and 237 nm	%RSD—0.53–2.45	*Talanta*, 42, 1631, 1995[91]
Fruit juice, supplements, urine	Solid phase spectrophotometry Reduction of Fe(III) complexed with ferrozine (chelate), chelate is sorbed onto Sephadex QAE A-25 anion-exchange gel, absorbance of gel at 567 nm and 800 nm is determined, $A = A_{567} - A_{800}$	%RSD—0.91	*Anal. Chim. Acta*, 360, 143, 1998[88]
Supplements, urine	Solid-phase UV spectrophotometry AA sorbed onto Sephadex QAE A25 anion exchange resin, resin packed in 1 mm cell, absorbance at 267 nm and 400 nm determined, $A = A_{267} - A_{400}$	%RSD—0.74 DL—0.05 μg mL^{-1}	*J. Pharm. Biomed. Anal.*, 20, 247, 1999[93]
Supplements	SIA Oxidation of AA to DHA with cesium(IV) in sulfuric acid medium, decrease in absorbance followed at 410 nm	%Recovery—99–101	*Analyst*, 124, 917, 1999[94]
Supplements, candy, urine	FIA coupled with solid-phase spectrophotometry, AA retained on a 1 mm Sephadex QAE A-25 anion exchange gel layer in a quartz flow-through cell, absorbance of solid phase monitored at 267 nm	DL—0.02–0.04 μg mL^{-1} %RSD—0.87–1.08	*Fres. J. Anal. Chem.*, 363, 92, 1999[95]
Fruit juice, soft drinks	Polyaniline film coated on microwell plates, absorbance of films at 650 nm decreases when subjected to diluted sample extracts	DL—1 mg L^{-1}	*Anal. Chem.*, 72, 4296, 2000[96]
Pharmaceuticals	Second derivative UV spectrophotometry at amplitudes of 280 nm and 272 nm, alternatively TLC resolution with direct scanning at 260 nm	TLC %RSD—2.4 2nd Derivative %RSD—0.6	*Pharmazie*, 55, 10, 2000[97]
Supplements, milk, urine, vegetables	Reduction of Fe(III) to Fe(II) and formation of Fe(II)-4-(2-pyridylazo)resorcinol complex, extraction into *n*-butanol, measure at 710 nm	%CV—2.9 %Recovery—97–102	*Anal. Chim. Acta*, 427, 245, 2001[98]
Supplements	Reduction of Fe(III) to Fe(II), formation of Fe(II)-p-carboxyphenyl-fluorone complex in a cationic surfactant micellar medium, no extraction, measure at 655 nm	%Recovery—96–103	*Anal. Sci.*, 17, 853, 2001[99]

Supplements, drinks	Reduction of Fe(III) to Fe(II), formation of Fe(II)-pyridine-2,6-dicarboxylic acid complex, measurement at 470 nm	%Recovery—97–101	*Ann. Chim.*, 92, 1159, 2002[100]
Vitamin C tablets	SIA, throughput—60 h^{-1} Reduction of permanganate by AA followed by decreased color intensity at 525 nm	%RSD—2.9	*Talanta*, 58, 1139, 2002[101]
Supplements, orange juice	FI, throughput—100 h^{-1} Inhibition by AA of the oxidation of pyrogallol red by iodate in H_2SO_4 media, measure at 470 nm	%RSD—0.6–3.4	*Anal. Lett.*, 35, 909, 2002[102]
Rat tissues	FI, throughput—40 h^{-1} Reduction of Fe(III) to Fe(II), formation of Fe(II)-2,2′-dipyridyl complex, measure at 510 nm	%RSD—1.2	*J. Pharm. Biomed. Anal.*, 29, 755, 2002[103]
Tea	Inhibition of the oxidation of 1,5-bis (*p*-hydroxy benzaldene) thiocarbohydrazone by horseradish peroxidase by AA, linear relationship between ΔA_{386} and AA concentration	%Recovery—96–105	*J. Agric. Food. Chem.*, 51, 4198, 2003[104]
Fruits, vegetables, juices	Second order derivative at 267.5 nm, extraction with 1.0 M HCl	r^e—0.9993 DL—4.2×10^{-6} M	*Eur. Food Res. Technol.*, 217, 269, 2003[105]
Tablets, supplements	Reduction of Cu(II)-NH_3 complex to CU(I)-NH_3 complex by AA, measure absorbance at 600 nm	%Recovery—93–101 %RSD—1.0–5.0	*J. Anal. Chem.*, 58, 927, 2003[106]
Foods, pharmaceuticals	FIA, throughput—110 h^{-1} Two solid phase reactors Reactor 1—30% m/m solid I_2 suspended on silica gel beads Reactor 2—silica gel AA is oxidized to DHA in reactor 1 (sample blank) AA is determined after reactor 2, measured at 267 nm, difference in analytical signal between the reactors is proportional to AA content	%RSD < 110 DL—0.08 μg mL^{-1}	*Talanta*, 61, 173, 2003[107]
Pharmaceuticals	SIA, throughput—26 h^{-1} Simultaneous determination of rutin and AA Analytes resolved on C_{18} SPE column, AA and rutin measured at 262 nm	%RSD—0.7	*Anal. Chim. Acta*, 497, 165, 2003[108]
Supplements, foods	FIA Reduction of Fe(III)-thiocyanate complex to Fe(II) + SCN^- by AA, unreacted complex is measured at 462 nm	%Recovery—97–103 DL—0.36 mg mL^{-1} %RSD—2.0	*Acta Chim. Slov.*, 51, 717, 2004[109]

Continued

Table 5.6 (Continued)

Matrix	Description	Accuracy/precision	References
Pharmaceuticals	Cyclic FIA, throughput—60 h^{-1} Reduction of Fe(III) to Fe(II) and complex formation of Fe(II) with 1,10-phenanthroline with the subsequent oxidation of the Fe(II) complex to the Fe(III) complex by peroxodisulfate. The Fe(II) complex is measured at 510 nm prior to cycling back to Fe(III)	%RSD—0.3–1.2 %Recovery—96–104	*Talanta*, 64, 1273, 2004[110]
Juices, supplements, sweets	Bead injection spectroscopy—FIA, Sephadex QAE A-25 is injected and immobilized on the beads, AA in sample extract reduces Prussian Blue to Prussian White, decrease in absorbance at 720 nm, beads are discarded by reversing flow	%RSD—5.0 DL—4.5×10^{-7} M	*Microchem. J.*, 78, 157, 2004[111]
Model solutions, juices	A transparent polymethacylate matrix modified with 2,2′-dipyridyl and Fe(III) was used as a sensor for AA, the solid phase plates after exposure to AA are measured against a nonreacted plate spectrophotometrically or visually compared to reference plates Reduction of Fe(III) complex to the Fe(II) complex results in a color change of yellow to reddish pink	—	*J. Anal. Chem.*, 59, 871, 2005[112]
Tablets, powders	AA dissolved in methanol, UV at 245 nm	—	*J. Pharm. Biomed. Anal.*, 36, 1107, 2005[113]
Fruit juice, red wine, supplements	Reduction of Cu(II) to Cu(I) with formation of 2,9-dimethyl-1,10-phenanthroline-Cu(I) chelate, determine absorbance at 450 nm	%RSD—3	*Talanta*, 65, 1226, 2005[114]
Fruit juice, supplements	Reduction of polyaniline film, absorbance at 700 nm	%CV—0.8–2.3	*Talanta*, 65, 1045, 2005[115]
Fluorometric			
Foods	Robotic extraction with MPA:HAC, C_{18} SPE, OPD, FIA, derivatization, Ex λ = 350, Em λ = 430	%Recovery—69–112	*J. Micronutr. Anal.*, 6, 109, 1989[116]
Wine, beer, pharmaceuticals, urine	AA oxidation in the presence of laccase to DHAA is monitored by the formation of the OPP quinoxaline derivative, FIA, Ex λ = 360, Em λ = 430	%RSD—2.0–4.1 %Recovery—88–104 QL—0.025 μg mL^{-1}	*Anal. Chim. Acta*, 309, 271, 1995[117]

Model system	Enhancement by AA of the fluorescence of the mimetic enzyme for peroxidase-catalyzed oxidation of p-cresol by β-cyclodextrin-hemin, Ex λ = 322, Em λ = 418	DL—3.45×10^{-10} mol L^{-1}	*Anal. Chim. Acta*, 413, 167, 2000[118]
Fruit juice, soft drinks, serum, supplements	Stopped-flow mixing, oxidation of AA to DHAA, OPD derivatization, Ex λ = 366, Em λ = 435	%RSD—0.5	*Analyst*, 126, 1436, 2001[119]
Vitamin C tablets	Enhancement of fluorescence of the product of the reaction of AA with 2,3-diaminonaphthalene by β-cyclodextrin, Ex λ = 400, Em λ = 520	DL—8 mg mL^{-1}	*Anal. Lett.*, 34, 1331, 2001[120]
Vitamin C tablets and injectables	Activation by AA of the mimetic enzyme for peroxidase-catalyzed oxidation of p-cresol by hemoglobin, Ex λ = 318, Em λ = 422	DL—3×10^{-10} M %RSD—1.6	*Anal. Sci.*, 18, 977, 2002[121]
Vitamin C tablets	Condensation reaction between AA and OPD in the absence of oxidant at pH 9.4, Ex λ = 360, Em λ = 430	DL—0.006 μg mL^{-1}	*Talanta*, 59, 95, 2003[122]
Supplements, model systems	FIA of AA and thiamin, OPD derivative of AA, thiochrome derivative of thiamine, Ex λ = 356, Em λ = 440 throughput—25 h^{-1}	%RSD—0.5–0.6 DL—1.3 μg mL^{-1} QL—3.9 μg mL^{-1}	*J. Pharm. Biomed. Anal.*, 34, 551, 2004[123]
Chemiluminescence			
Fruit juice, supplements	AA reduction of Fe(III) to Fe(II) monitored by Fe(II) catalyzed light emission from luminal oxidation by H_2O_2, FIA	%RSD—1.4 DL—1×10^{-6} mol L^{-1}	*Analyst*, 118, 639, 1993[124]
Fruit juice, soft drinks, supplements, blood	When irradiated by visible light, AA oxidation products are photosensitized by toluidine blue that react with lucigenin by yield strong chemiluminescence, FIA	%RSD—1.22 DL—2×10^{-10} M QL—0.17 mg mL^{-1}	*Anal. Chim. Acta*, 308, 299, 1995[125]
Vitamin C tablets	Reduction of Cu(II) by AA to Cu(I), production of H_2O_2 and oxygen-free radicals sensitizes the chemiluminescence of luminol, FIA	DL—5.8×10^{-8} mol L^{-1}	*Anal. Sci.*, 16, 1317, 2000[126]
Fruit juice	AA in the presence of hexacyanoferrate(III) with luminol yields chemiluminescence, background correction was made by destroying AA with L-ascorbate oxidase immobilized porous glass, two chemiluminescence signals (with and without L-ascorbate oxididase treatment) corrected for extract interferences, FIA	%RSD—3.13	*Luminescence* 15, 305, 2000[127]

Continued

Table 5.6 (Continued)

Matrix	Description	Accuracy/precision	References
Vegetables	AA catalysis of the chemiluminescence of Rhodamine B with cerium (IV) in sulfuric acid media	%RSD—0.92 DL—1.0×10^{-13} mol L^{-1}	*Anal. Chim. Acta*, 464, 289, 2002[128]
Vegetables, vitamin C tablets	Chemiluminescence from oxidation of AA with permanganate immobilized on anion exchange resin is a flow-through sensor, FIA, throughput—120 h^{-1}	%RSD—1.6	*Anal. Lett.*, 36, 2783, 2003[129]
Vitamin C tablets	Chemiluminescence from oxidation of AA with acidic potassium permanganate on soluble Mn(IV) were compared by FIA and SIA	Potassium permanganate DL (FIA)—5×10^{-8} M DL (SIA)—5×10^{-8} M Mn(IV) DL (FIA)—1×10^{-8} M DL (SIA)—5×10^{-9} M	*Talanta*, 64, 130, 2004[130]
Soft drink powder	Quenching of chemiluminescence due to AA in an iron-chlorophyll in aqueous solution mixed with a Me CN/water solution containing H_2O_2, FIA	%RSD—3.8	*Anal. Sci.*, 21, 579, 2005[131]
Supplements	Reduction of Fe(II) to Fe(II) by AA, Fe(II) detected by chemiluminescence of luminol-(Fe II)-O_2 system, FIA	%RSD—3.2	*Microchim. Acta*, 149, 205, 2005[132]

Table 5.7 Electrochemical Methods for Analysis of Vitamin C

Matrix	Method description	Accuracy/precision	References
Dopamine injectables	CME (chemically modified electrode) A poly (malachite green) modified glass carbon disk electrode was the working electrode with a platinum slice as a counter electrode and a SCE as the reference electrode, anodic peaks of AA and dopamine were separated at the modified electrode allowing the determination of dopamine in the presence of AA	%RSD—3.4 for dopamine %Recovery—98–103 for dopamine	*Anal. Sci.*, 15, 41, 1999[133]
Beverages, fruit juice	CME 1,5,8,12-Tetraaza-2,4,9,11-tetramethylcyclotetra-decainatonickel(II) was electrolyzed on a glassy carbon electrode, amperiometric analysis of AA was at 0.5V vs. Ag/AgCl/NaCl, FIA	%Recovery—93–121 %RSD—5	*J. Elelectroanal. Chem.*, 468, 85, 1999[134]
Fruit juice, supplements	CME Glassy carbon modified with cellulose acetate film containing 2,6-dichlorophenol, AA monitored at + 100 mV versus Ag/AgCl/3 M KCl, pH 6.5, FIA, throughput = 25 h^{-1}	%Recovery—92–110 %CV—0.7–1.2	*Anal. Chim. Acta*, 409, 113, 2000[135]
Fruit juice	CME Inclusion complex of ferrocene with β-cyclodextrin was used to prepare a modified carbon paste electrode, AA was oxidized at the the electrode in NH_3-NH_4Cl, pH 10.0, anodic peak potential = +0.20 V versus SCE	DL—1.0×10^{-7} mol L^{-1} %Recovery—100–108 %RSD—0.47–1.53	*Talanta*, 51, 1019, 2000[136]
Model system	CME Carbon paste electrode containing ruthenium (III) diphenyldithiocarbamate, CME (working electrode), platinum foil (counter electrode), SCE (reference electrode), cyclic voltammogram showed anodic peak at 0.38 V and cathodic peak at +0.22 V versus SCE	—	*Anal. Chim. Acta*, 405, 93, 2000[137]
Model system	CME Carbon paste electrode spiked with ferrocene carboxylic acid, AA oxidation at the CME versus Ag/AgCl/KCl occurred at 248 mV less than at an unmodified electrode	DL—1.08×10^{-5} M	*J. Electroanal. Chem.*, 515, 45, 2001[138]
Supplements, fruit juice, wine	CME Carbon paste electrode containing aza macrocycles, best response was obtained with 1,4,7-triosyl-1,4,7-triazacyclononame, differential pulse volammetry was used with CME in buffer containing 0.05 M Zn $(NO_3)_2$	DL—0.1 μg mL^{-1}	*Anal. Chim. Acta*, 439, 291, 2001[139]

Continued

Table 5.7 (Continued)

Matrix	Method description	Accuracy/precision	References
Model system	CME Wax saturated graphite electrode surface was treated with glutamic acid, cyclic voltammograms of provided oxidation peaks for dopamine and AA separated by 200 mV	DL (mol L^{-1}) dopamine—1.8×10^{-6} AA—1.3×10^{-5}	*Anal. Lett.*, 34, 1585, 2001[140]
Fruit juice, vegetable juice, supplements serum	Film Modified Electrode A1 electrode was modified with nickel hexcyanoferrate, cyclic or linear sweep voltammetry in phosphate buffer, pH 7.2	%RSD <5 DL—2×10^{-6} M	*Electroanal.*, 14, 206, 2002[141]
Model system	CME Modified electrodes were constructed by intercalating carbon manotubes on a graphite surface, voltammic separation of dopamine and AA were separated by 270 mV (anodic) by cyclic voltammetry	—	*Analyst*, 127, 653, 2002[142]
Fruit juice	CME A polyviologen-modified glassy carbon electrode was used to directly determine AA in deeply colored, viscous fruit juices, anodic oxidation peak was at +0.22 V (versus Ag/AgCl)	%Recovery—100.9–98.4	*Electroanal.*, 14, 1597, 2002[143]
Juice, vitamin C tablets, urine	CME A silica sol-gel glass-coated ferricyanide-doped Tosflex-modified screen electrode was used for mediated oxidation of AA, FIA at 0.3 V (versus Ag/AgCl), pH 7.0 used a flow rate of 0.5 mh min^{-1}	%RSD—2.68 DL—46 nM	*Electroanal.*, 15, 1171, 2003[144]
Fruit juice, vitamin C tablets	Voltammetric assay of AA using methylene blue as a mediator with a glass carbon electrode, glassy carbon (working electrode), platinum (counter electrode), Ag/AgCl (reference electrode), optimum conditions at –0.04 V versus Ag/AgCl	%RSD—1.6–1.9 LD—1×10^{-6} M	*Anal. Lett.*, 36, 591, 2003[145]
Vitamin C tablets, injectables, foods	CME A glassy carbon electrode containing $Ni(Me_2(CH_3CO)_2[14]tetraenoN_4)$ complex was used for cyclic voltametric assay of AA, CME (working electrode), platinum wire (counter electrode), Ag/AgCl (reference electrode), use of the CME shifted peak potential negative by 205 mV	%RSD <2.47 DL—3.1×10^{-7} M	*Anal. Sci.*, 19, 1671, 2003[146]

Model system	CME A ferrocene modified carbon paste electrode was applied to the measurement of AA by cyclic voltammetry, double-step chronoamperometry and chronocoulometry, CME (working electrode), platinum wire (counter electrode), $Ag/AgCl/KCl_{sat}$ (reference electrode)	DL—3.2×10^{-5} M	*Bull. Electrochem.*, 19, 17, 2003[147]
Multivitamin tablets	Simultaneous analysis of AA and riboflavin using a glassy carbon electrode, oxidative peaks were at +0.35 V and –0.47 V for AA and riboflavin versus SCE	%RSD riboflavin—2.6 AA—3.1	*J. Trace Microprobe Tech.*, 21, 111, 2003[148]
Supplements	A flow through electrolytic cell composed of a gold and copper-modified electrode polarized at –0.1 and 0.5 V, respectively, was used for the amperometric assay of AA and glucose, copper-modified and gold disk (working electrode), platinum wire (counter electrode), Ag/AgCl (reference electrode), FIA	—	*Electroanal.*, 15, 1884, 2003[149]
Vitamin C tablets, dopamine injectables, model systems	CME A polyphenosafranine film modified glassy carbon electrode was used to simultaneously assay AA, dopamine, and serotonin by cyclic and differential pulse voltammetry, CME (working electrode), platinum foil (counter electrode), SCE (reference electrode)	%RSD—1.4–1.5	*Elelectrochem. Comm.*, 5, 667, 2003[150]
Urine	CME A gold electrode was modified with a film of hydrated osmium complex through electrodeposition, oxidation potential of AA increased and shifted negatively to 0.01 V versus SCE with the CME	LD—0.6 µM	*Electroanal.*, 16, 319, 2004[151]
Brain tissue	CME Simultaneous assay of AA and dopamine with poly(toluidine) blue film modified glassy carbon electrode, CME (working electrode), platinum wire (counter electrode), SCE (reference electrode)	%RSD—1.5 for AA and 0.42 for dopamine	*Anal. Sci.*, 20, 1725, 2004[152]
Supplements	CME Carbon paste electrode was modified with calixarenes for cyclic and differential pulse voltammetric assay of AA, CME (working electrode), gold (counter electrode), SCE (reference electrode)	DL—30 ppb	*Electroanal.*, 16, 2082, 2004[153]

Continued

Table 5.7 (Continued)

Matrix	Method description	Accuracy/precision	References
Model system	CME An acetate/choline modified glassy carbon electrode was used for the simultaneous assay of AA, dopamine and serotonin, CME (working electrode), platinum wire (counter electrode), SCE (reference electrode)	DL—9×10^{-7} M	*J. Electroanal. Chem.*, 569, 135, 2004[154]
Model system	CME A gold electrode with a film of ferrocene appended poly(allylamine HCl) and poly(potassium vinyl sulfate) was used to monitor oxidation of AA, CME (working electrode), platinum wire (counter electrode), Ag/AgCl (reference electrode), cyclic voltammetry and amperometry	LD—0.1 mmol L^{-1}	*Anal. Bioanal. Chem.*, 380, 98, 2004[155]
Vitamin C tablets and drops	The oxidation of hydroquinone to *p*-benzoquinone by horseradish peroxidase was mediated by the reduction of AA and monitored by a glassy carbon electrode at –0.15 V, FIA using continuous flow/stopped flow/continuous flow, throughput = 20 h^{-1}	%Recovery—99–101 LD—6 nm	*Talanta*, 64, 1009, 2004[156]
Serum	CME A carbon paste electrode was modified with cobalt (II)-4-methylsalophen as a Schiff base complex, anodic oxidation of AA and cysteine was monitored by cyclic and differential pulse voltammetry, CME (working electrode), platinum wire (counter electrode), Ag/AgCl (reference electrode)	%RSD ≤ 2.5	*Electrochim. Acta*, 50, 77, 2004[157]

Table 5.8 Applications of Capillary Electrophoresis to Analysis of Vitamin C

Matrix	Description	Accuracy/precision	References
Model system injectables	MECC 80 cm × 100 μm, 0.02 M phosphate containing 0.05 M SDS, +30 kV, 20 μA, 254 nm	%RSD < 2.1	*J. Chromatogr.*, 447, 133, 1988[162]
Fruit juice, supplements	CZE 20 cm × 25 μm, 0.1 M phosphate, pH 5.0, +8 kV, 265 nm	DL—0.5 μg ML^{-1}	*J. Pharm. Biomed. Anal.*, 10, 717, 1992[163]
Fruits	CZE 40 cm × 100 μm, 20 mM phosphate, pH 7.0, +6 kV, 60 μA, 254 nm	Results comparable to LC	*J. Chromatogr.*, 645, 197, 1993[164]
Biological fluids, fruit beverages	CZE 30 cm × 75 μm, 100 mM tricine, pH 8.8, 254 nm	%RSD—1.9–3.3 %Recovery—98	*J. Chromatogr.*, 633, 245, 1993[165]
Supplements	CZE 40 cm × 50 μm, 20 mM phosphate, pH 7.0, +20 kV, 215 nm	—	*J. Chromatogr. A*, 652, 495, 1993[166]
Supplements	CZE 51 cm × 75 μm, 0.02 M Na phosphate, pH 9.0, +6.0 kV, 254 nm	—	*J. Chromatogr.*, 636, 133, 1993[167]
Supplements, fruit juices	CZE 60 cm × 50 μm, 20 mM phosphate, pH 8.0, +30 kV, DAD, on-line	%RSD—2.5	*J. Chromatogr. A*, 717, 255, 1995[168]
Organic acids including AA	MECC 43 cm × 75 μm, 10 mM phosphate, pH 8.0, +20 kV, scan absorbance (200–350 nm)	DL—2.0×10^{-6} M %RSD <3	*J. Chromatogr. A*, 716, 291, 1995[169]
Fruits, vegetables	MECC 40 cm × 75 μm, 0.05 M Na deoxycholate, 0.01 M borate, 0.01 M KH_2PO_4, pH 8.6, +25 kV, 254 nm	%CV—0.5–3.4 Data comparable to LC	*Food Chem.*, 53, 43, 1995[170]
Beer, wine, fruit drinks	MECC 100 cm × 75 μm, 0.05 M SDS in 0.005 M K_2HPO_4, pH 9.2, or 0.05 MM Na deoxycholate (dissolved in 1:1 mixture of 0.02 M KH_2PO_4 and 0.02 M Na tetraborate), pH 8.6, or cetyltrimethylammonium bromide (0.05 M) (dissolved in 1:1 mixture of 0.02 M KH_2PO_4 and 0.02 M Na borate, pH 8.6, +25 kV, 254 nm	%CV—0.5–3.8	*J. Chromatogr. Sci.*, 33, 426, 1995[171]
Plant tissue	CZE 57 cm × 75 μm, 200 mM borate, pH 9.0, +25 kV, online DAD (260 nm optimum)	DL—84 fmol $injection^{-1}$	*Anal. Biochem.*, 239, 8, 1996[172]

Continued

Table 5.8 (Continued)

Matrix	Description	Accuracy/precision	References
Supplements	CZE 48.5 cm × 50 μm, 0.05 M Na tetraborate, 0.05 M Na citrate, pH 8.5, +25 kV, 225 nm	%RSD—1.0–2.9 %Recovery—98.5–100.9	*J. Pharm. Biomed. Anal.*, 15, 1113, 1997[173]
Qiangli Yingqiao	CZE 53 cm × 75 μm, 20 mmol L^{-1} Na tetraborate + 20 mmol L^{-1} NaH_2PO_4, pH 7.2, +25 kV, 254 nm	%RSD—2.10	*J. Liq. Chrom. Rel. Technol.*, 20, 3245, 1997[174]
Vegetables	CZE 37.5 cm × 50 μm, 20 mM Na tetraborate, pH 9.2, +20 kV, 270 nm	%RSD—8.7 %Recovery—97–112	*J. Chromatogr. A*, 772, 313, 1997[175]
Vegetables, fruits, juices	CZE 27 cm × 57 μm, 100 μM Na borate, pH 8.0, +15 kV, 245 nm	%CV < 0.5 %Recovery—95–98.6	*J. Chromatogr. A*, 781, 435, 1997[176]
Plant tissue	CZE 33.5 cm × 50 μm, 60 mM NaH_2PO_4, and 60 mM NaCl containing 0.0001% hexadimethonium, pH 7.0, –15 kV, 265 nm	DL—0.02 μg ML^{-1} $\%RSD_r$—0.9 $\%RSD_R$—1.2	*Anal. Biochem.*, 265, 275, 1998[177]
Fish feed, fish plasma, fish tissue	CZE (L-ascorbyl-2-phosphate, -2-diphosphate, -2-triphosphate, -2-sulfate) 48.5 cm × 150 μm, 80 mM tricine, pH 9.2, +30 kV, 100–150 μA, 254 nm	$\%RSD_r$—2.8	*J. Chromatogr. B*, 715, 369, 1998[178]
Juice, supplements	MECC 64.5 cm × 50 μm, phosphate, cholate, 1-propanol buffers at various concentrations, +10-22 kV, 214 and 255 nm	%RSD—6	*J. Chromatogr. A*, 802, 233, 1998[179]
Foods, model systems	MECC (synthetic antioxidants and AA) 60 cm × 75 μm, 40 μM Na cholate, 15 mM SDS, 10 % MeOH, 10 mM borate, pH 9.3, +18 kV, 254 nm	—	*J. Agric. Food Chem.*, 47, 1970, 1999[180]
Plasma, aqueous humor	CZE 47 cm × 75 μm, 50 mM tricine, pH 8.8, +12 kV, 254 nm	%CV—1.27 %Recovery—98.8	*Clin. Biochem.*, 32, 473, 1999[181]
Supplements	MECC, CZE 48.5 cm × 50 μm, CZE buffer (0.05 M Na tetraborate adjusted to pH 8.5 with boric acid), MECC buffer (0.05 Na tetraborate adjusted to pH 8.5 with boric acid containing 25 mM SDS, +25 kV, 225 nm	$\%RSD_r$ (migration time)—0.33 for MECC and 0.8–1.8 for CZE %Recovery—99.4–100.7 for MECC and 99.8–100.6 for CZE	*J. Chromatogr. A*, 853, 391, 1999[182]

Supplements	MECC 48.5 cm × 50 μm, 20 mM phosphate-borate containing 50 mM SDS, pH 9.0, +20 kV, 215 nm	DL—0.10 μg ML^{-1} %RSD—0.88	*Anal. Chim. Acta*, 396, 55, 1999[183]
Plant tissue	CZE 33.5 cm × 50 μm, 60 mM NaH_2PO_4, 60 mM NaCl containing 0.0001 % hexadimethrin bromide, pH 7.0, −15 kV, 265 nm	DL—0.20 μg ML^{-1} $\%RSD_r$—0.8 $\%RSD_R$—1.2	*Environ. Sci. Technol.*, 34, 1331, 2000[184]
Model systems	CZE 57 cm × 75 μm, 0.2 M borate, pH 9.0, +25 kV, 254 nm	—	*J. Agric. Food Chem.*, 48, 37, 2000[185]
Citrus juice	CZE 70 cm × 50 μm, 35 mM Na borate containing 5 % (V/V) MeCN, pH 9.3, +21 kV, 270 nm	QL—0.5 mg L^{-1}	*AOAC Intl.*, 84, 987, 2001[186]
Neurons, neuronal tissue	CZE 80 cm × 50 μm, 30 mM borate, pH 9.85, +20 kV, laser-induced fluorescence, derivatization of AA and DHA with 4,5-dimethyl-1,2-phenylenediamine	—	*Anal. Chem.*, 74, 5614, 2002[187]
Lycopersion fruit	CZE 27 cm × 50 μm, 400 mM borate containing 0.02 % Hexadimethrine bromide, pH 8.0, −15 kV, 254 nm	LD—0.2 μg ML^{-1} $\%RSD_{Intra}$—0.5 $\%RSD_{Inter}$—2.0	*Anal. Biochem.*, 296, 218, 2001[188]
Plasma	CZE 60 cm × 75 μm, 100 mM tricine, pH 8.8, +17 kV, 254 nm	%CV—1.5–3.6	*J. Food Drug Anal.*, 12, 217, 2004[189]
Urine, serum	Microchip CE Separation channel 5 cm × 30 μm ×10 μm, 20 mM phosphate, pH 7.5, +1.5 kV, EC detection (+1.0 V vs. Ag/AgCl)	DL—0.71 μM %RSD—1.5–2.2	*Talanta*, 64, 750, 2004[190]
Fruit juice	CZE 55 cm × 75 μm, 80 mmol L^{-1} boric acid, 5 mmol L^{-1} boxax, pH 8.0, 270 nm	%RSD—2.7–3.0 %Recovery—97	*Talanta*, 65, 794, 2005[191]
Beverages	Microchip CE Separation channel—85 mm × 50 mm, 10 mM histidine/0.135 mM tataric acid, 0.1 mM CTAB, 0.25% hydroxypropyl-β-CD; pH > 5.0, −5 kV, C^4 D detection	DL—3 mg L^{-1} %RSD—1.8	*Electrophoresis*, 26, 4648, 2005[192]
Cosmetics	MECC 44.5 cm × 50 μm, 10 mM borate containing 50 mM SDS, pH 9.5, +20 kV, 265 nm	$\%RSD_{Inter}$—1.13 $\%RSD_{Intra}$—0.62	*Anal. Chim. Acta*, 576, 124, 2006[193]
Grapefruit peel and juice	CZE 75 cm × 25 μm, 60 mM borate, pH 9.0, +0.95 kV, EC detection (working electrode—carbon disc, auxillary electrode—Pt, reference electrode—SCE)	DL—1.0×10^{-6} g mL^{-1}	*Food Chem.*, 100, 1573, 2007[194]

Table 5.9 Selected Liquid Chromatographic Methods for the Analysis of Vitamin C

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Various foods/ AA, IAA	Blend w/0.3 N TCA containing 0.5 mL octanol per 60 mL. Add NaOAC buffer and ascorbate oxidase, incubate, 37°C. Add 1% OPD, incubate, 37°C, 30 min	Sperisorb ODS-2, 5 µm, 12.5 cm × 4 mm. Mobile phase—isocratic 0.08 M KH_2PO_4:MEOH (80:20), pH 7.8 Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 365 Em λ = 418	QL—1.5–3.4 mg 100 g^{-1} %Recovery—97.1	*J. Agric. Food Chem.*, 32, 352, 1984[82] J. *Food Comp. Anal.*, 7, 252, 1994;[83] 8, 12, 1995[84]
Various foods/ AA, DHAA, IAA, DHIAA	Blend w/0.1 M citric acid containing 5 µM EDTA plus equal volume of HEX. Centrifuge. Dilute 1:1 w/mobile phase, or blend w/MPA-HAC solution containing EDTA	Altex ODS, 5 µm, 25 cm × 4.6 mm Three in series Mobile phase—isocratic 0.1 M NaH_2PO_4 containing 5 mM EDTA and 5 mM TBAP, pH 5.0 Flow rate—0.5 mL min^{-1}	Postcolumn oxidation w/OPD Fluorescence Ex λ = 350 Em λ = 430	%Recovery—90–107	*J. Chromatogr. Sci*, 22, 485, 1984[196] *J. Micronutr. Anal.*, *1*, 143, 1985;[78] 4, 109, 1988;[197] 6, 109, 1989[79] *J. Food Comp. Anal.*, 3, 105, 1990[198] *J. Nutr. Biochem*, 4, 184, 1993[199]
Plasma, cured meats	Blend or mix w/5 or 10% MPA. Homogenize meat or mix plasma. Centrifuge. Dilute 10× w/mobile phase	Altex ODS Ultra sphere, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic 0.04 M NaOAC, 0.005 M TBAP containing 0.2 mg mL^{-1} EDTA, pH 5.25 Flow rate—0.4 or 0.8 mL min^{-1}	EC, glassy carbon, +0.6 V vs Ag/AgCl	%Recovery—90–107 DL (on-column)—0.25 ng	*J. Liq. Chromatogr.*, 8, 31, 1985[200] *J. Food Sci.*, 52, 53, 1987[201]
Various foods, animal tissues/ AA, DHAA, IAA, DHIAA	Homogenize in MPA: HAC (30 g + 80 mL) diluted to 1 L or 0.1 M citric acid. Dilute fruit juice w/mobile phase	PLRP-S, 2 columns, in series, 5 µm, 15 cm × 4.6 mm, 25 cm × 4.6 mm Mobile phase—isocratic 0.2 M NaH_2PO_4, pH 2.14 Flow rate—0.5 mL min^{-1}	220 nm or postcolumn oxidation w/$HgCl_2$ and OPD derivatization, Fluorescence Ex λ = 350 Em λ = 430	DL (on-column)—1.6 µg (Fluorescence) %Recovery—95–105	*J. Chromatogr.*, 437, 447, 1988[202] *Food Chem.*, 28, 257, 1988[203] *J. Micronutr. Anal.*, 7, 67, 1990[204]

Various, foods/ AA, DHAA	Homogenize w/10- to 50- fold excess of 20 mM H_2SO_4. For DHAA, reduce to AA w/DTT	Sulfonated polystyrene divinyl benzene, 5 µm, 10 cm × 4.6 mm Mobile phase—isocratic 20 mM H_2SO_4 Flow rate—0.6 mL min^{-1}	EC, P+ electrode, +0.6-0.8 V versus Ag/AgCl	DL (on-column)—0.1 ng %Recovery—102	*J. Assoc. Off. Anal. Chem.*, 72, 681, 1989[205]
Various foods/AA	Homogenize in MPA: HAC (3% MPA in 8% HAC)	µBondapak C_{18}, 10 µm, 30 cm × 3.9 mm Mobile phase—isocratic MeOH:water (55:45) Flow rate—1 mL min^{-1}	Precolumn oxidation w/Norit, OPD derivatization, Fluorescence Ex λ = 350 Em λ = 436	QL—0.6 mg mL^{-1} DL—10 mg 100 μL^{-1} %Recovery—90–108	*J. AOAC Int.*, 75, 887, 1992[206]
Diets/AA, Juices/ AA, DHAA	Mix w/6% MPA, filter for juices, dilute to 10 µg mL^{-1} w/water, add 10 µL α-methyl-DDPA and 800 µL of 2% MPA to 20 µL (AA analysis). For DHAA, reduce to AA w/cysteine	Intensil 0 DS-2, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic 100 mM KH_2PO_4 containing 1 mM EDTA, pH 3.0 Flow rate—0.6 mL min^{-1}	EC, +70 mV, Ag/AgCl or 300 mV, Ag/AgCl	QL—9.6 mg 100 g^{-1} %Recovery—97–98	*J. Chromatogr.*, 606, 277, 1992[207] *J. Chromatogr. A*, 654, 215, 1993[208]
Various foods/AA, DHAA, IAA, DHIAA	Homogenize in 17% MPA, centrifuge, filter, reduce oxidized forms to AA and IAA w/ homocysteine	Supelcosil LC-18 DB 5 µm, 25 cm × 4.6 mm Three columns in series Mobile phase—isocratic 0.08 M NaOAC, pH 5.4 w/5 µM TBAS and 0.15% MPA Flow rate—1 mL min^{-1}	EC Glassy carbon vs Ag/AgCl, 0.6 V	DL (on-column)—0.5 ng %Recovery—94–107 for AA and 94–108 for IAA	*J. Liq. Chromatogr.*, 15, 753, 1992[209] *J. Food Comp. Anal.*, 3, 3, 1990;[210] 7, 158, 1994[211]

Continued

Table 5.9 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Food, tissue/AA, DHAA, IAA, DHIAA	Homogenize in 17% MPA, centrifuge, filter, mix 500 µL w/115 µL 45% KH_2PO_4, pH 9.8. Final pH = 7.1. Add 0.85% MPA to 2 mL. Add 100 µL–10 mL mobile phase. For DHAA and DHIAA assay, reduce w/homocysteine	PLRL-S, 5 µm, 25 cm × 4.6 mm Two columns in series Mobile phase—isocratic 20 mM NaH_2PO_4 containing 0.17% MPA, pH 2.6 Flow rate—0.6–0.7 mL min^{-1}	EC Glassy carbon versus Ag/AgCl, +0.7 V	DL (on-column)—0.5 ng QL—0.5 ng 20mL^{-1} %Recovery—94–104	*J. Liq. Chromatogr.*, 17, 2445, 1994[212]
Biologicals / L-AA, D-AA	Serum was diluted w/MPA and stored at –70°C	Cap Cell Pak NH_2 40°C Mobile phase—isocratic KH_2PO_4 (0.68 g) + Water (200 mL) + MeCN (800 mL) + H_3PO_4 (7.5 mL) Flow rate—1 mL min^{-1}	EC +7.00 mV	—	*J. Chromatogr. B*, 690, 25, 1997[214] *Clin. Chem.*, 34, 2217, 1988[213]
Fish feed, tissue/AA, DHAA, AMP, APP, AMS	Extract w/0.2 mol L^{-1} NaOAC, pH 4.8, deproteinize w/ perchloric acid, reduce DHAA to AA w/DTE. Ascorbate oxidase treatment was used to correct for background. Ascorbate phosphates were hydrolyzed to AA by acid phosphatase	Vydac C_{18}, 5 µm, 25 cm × 4.6 mm. Mobile phase—isocratic 0.04 M NaOAC, 0.05 mM EDTA, 0.5 mM TBAP, pH 3.76. Mix 24 mL MeOH w/1000 mL Flow rate—0.6 mL min^{-1}	250 nm	%Recovery—96–96	*Chromatographia*, 48, 43, 1998

Food, biologicals/ AA, DHAA, IAA, DHIAA	Homogenize 25–30 g into 130–170 mL 1% MPA w/0.5% oxalic acid, pH 2.0, under CO_2. Dilute to 250 mL, centrifuge, and filter aliquot	Jupiter C_{18}, 5 μm, 25 cm × 4.6 mm. Mobile phase—isocratic 2.3 mM DTMAC and 2.5 mM EDTA in 66 mM phosphate-20 mM acetate buffer, pH 4.5	AA, IAA 247 nm or by EC DHAA DHIAA Postcolumn OPD derivatization Fluorescence Ex λ = 350 Em λ = 430	%RSD_r—2.4–5.8%	*J. Chromatogr. B*, 730, 101, 1999[216]
Biologicals/AA, DHAA	Plasma, serum, urine—dilute 1:4 (90% MeOH in water w/ 1 mM EDTA). Cool, centrifuge, store, supernatant at –70°C	Ultrasphere ODS-DABS C_{18}, 5 μm, 25 cm × 4.6 mm or equivalent Mobile phase—isocratic 24–60% MeOH w/0.05 M NaH_2PO_4, 0.05 M NaOAC, 189 μM DTMAB, 36.6 μM TOAB, pH 4.8 Flow rate—1 mL min^{-1}	EC Coulometric +0.00 V—Detector 1 +0.25 V—Detector 2	—	*Meth. Enzymol.*, 299, 65, 1999[217]
Green beans/AA	Extract 15 g w/30 mL 4.5% MPA, filter, dilute to 50 mL w/water	Sphere clone ODS, 5 μm Mobile phase—isocratic 1.8 mM H_2SO_4, pH 2.6 Flow rate—0.9 mL min^{-1}	245 nm	Repeatability %CV—6.4–7.1	*Eur. Food Res. Technol.*, 210, 220, 2000[218]
Multivitamin-mineral tablets/ AA	Extract w/100 mL solution containing 1 g pyrogallol and 19.21 g citric acid per L. Filter extract through Costar Spin-X filter unit (0.45 μm) for 1 min	Hypersil BDS-C_{18}, 5 μm, 25 cm × 4.0 mm, Mobile phase—isocratic Ion-pair solution:MeCN (98:2) Ion-pair solution—10 mM 1-hexane sulfonic acid, 10 mL HAC, 1.3 mL TMA in water to 1 L Flow rate—1 mL min^{-1}	275 nm	%Recovery—92–100 %RSD—0.8–3.8	*J. Pharm. Biomed. Anal.*, 25, 985, 2001[219]
Honey/AA	Dissolve 10 g in 0.25% MPA	Shimadzu ODS, C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:water (15:85), pH 2.5 Flow rate—0.9 mL min^{-1}	254 nm	%Recovery—92–95	*J. Liq. Chrom. Rel. Technol.*, 24, 1015, 2001[220]

Continued

Table 5.9 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Sausage/AA	Extract 5 g w/20 mL 50% MPA w/1 mL EDTA, dilute to 50 mL w/water, centrifuge, filter	Spherisorb NH_2, 5 Mm, 25 cm × 4 mm Mobile phase—isocratic 0.02 M KH_2PO_4:MeCN (40:60), pH 3.6 Flow rate—1 mL min^{-1}	248 nm	DL—1.6 mg 100 g^{-1} %Recovery—91–96	*J. Food Prot.*, 65, 1771, 2002[221]
Beverages/AA	Direct injection after homogenization and dilution w/water, if necessary	Kromasil NH_2, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic 0.1 M HAC in water Flow rate—1.5 mL min^{-1}	250 nm	DL—1.2 mg L^{-1} QL—4.0 mg L^{-1} %RSD—2.1 ± 1.5	*Food Chem.*, 79, 141, 2002[222]
Water/AA	Water dilutions	Ion chromatography Dionex IonPac, 25 cm × 4 mm Mobile phase—isocratic 1.8 mM sodium carbonate—1.7 mM sodium hydrogen carbonate w/4 µM BTA	Fluorescence of Ce (III) via postcolumn reaction w/Ce (IV) Ex λ = 256 Em λ = 354	DL—18 ppb	*J. Chromatogr. A*, 956, 77, 2002[223]
Fruit juice/AA	Dilute 1–10 mL w/water, add 1 mg DTT to each mL	Any 5 µm C_{18} silica-based column Mobile phase—isocratic KH_2PO_4 (0.05% w/v), pH 2.5 containing DTT (0.1% w/v)	254 nm	%RSD_r—9.5–14.7 %RSD_R—6.4–30.5	*J. AOAC Int.*, 86, 367, 2003[224]
Foods/AA	Dilute sample to contain 100 mg AA in 10 mL mobile phase containing 20 µM L-methionine. Dilute to AA content of 1 µg mL^{-1} w/mobile phase	Intersil ODS, 5 µm, 15 cm × 3 mm Mobile phase—isocratic 0.2% H_3PO_4, pH 2.1 Flow rate—0.4 mL min^{-1}	EC +400 mV versus Ag/AgCl	%Recovery >90% DL (on-column)—0.1 ng	*Talanta*, 60, 1011, 2003[225]

Human lymphocytes/AA	Lymphocyte lysates were centrifuged at 18+ g for 10 min at 4°C. Dilute w/10% MPA containing 2 mM EDTA	End capped ODS-converted spherical silica CSC-Kromasil C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic 0.2 M KH_2PO_4/H_3PO_4, pH 3.0, containing 2 mM EDTA Flow rate—1 mL min^{-1}	245 nm	$\%CV_{Inter}$—0.25–9.98 $\%CV_{Intra}$—1.2–12.49	*Clin. Biochem.*, 38, 450, 2005[226]
Tropical fruits/AA, DDAA	Extract w/3% MPA—8% HAC	Shodex RSpak KC-811, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic 0.2% orthophosphoric acid Flow rate—1.2 mL min^{-1}	245 nm	DL—0.1 mg L^{-1}	*Food Chem.*, 96, 654, 2006[66]
Fatty fish/AA, DDAA	Extract w/4.5% MPA	Waters Symmetry C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:0.1% MPA (80:20) Flow rate—1 mL min^{-1}	Precolumn OPD or DMPD derivatization Fluorescence OPD derivatives Ex λ = 350 Em λ = 422 DMPD derivatives Ex λ = 362 Em λ = 444	%Recovery—92.4–93.0 for AA and 98.4–99.1 for DHAA	*Eur. Food Res. Technol.*, 223, 781, 2006[227]
Wine/AA	Filter—GHPAcrodisc, 25 mm, 0.45 µm Inject filtrate	PLRP-S, 100 Å, 5 µm, 15 cm × 4.6 mm Mobile phase—gradient Solvent A—water:TFA (99:1) Solvent B—MeCN 100% A—5 min To 100% B over 1 min Hold B for 4 min Back to A over 1 min	PDA 243 nm	%RSD Retention time <0.8 Red wine—2.2 White wine—1.5 DL—1 mg L^{-1} QL—5 mg L^{-1} %Recovery <90	*Anal. Chim. Acta*, 555, 242, 2006[228]
LC-MS					
Foods/AA	Extract 2 g w/5 mL MeOH and 25 mL of 3% MPA—8% HAC, filter. To 10 mL filtrate, add 15 mL 1% HAC	Waters Symmetry C_{18}, 3.5 µm, 7.5 cm × 4.6 mm coupled to an Atlantis dC_{18}, 5 µm, 15 cm × 2 mm Mobile phase—isocratic MeOH:HAC (70:30)	LC-MS-ESI Negative ion mode	DL—1.0 µg mL^{-1} QL—50 µg mL^{-1} %RSD—8.5 %Recovery—85	*J. Agric. Food Chem.*, 53, 7371, 2005[229]

and glacial acetic acid, sulfuric acid, and phosphoric acid are usually compatible to LC supports and mobile phases. If the assay requires quantitation of total ascorbic acid, the resolution system must be capable of resolving L-ascorbic acid from L-dehydroascorbic acid with use of a detection mode capable of detecting both forms of vitamin C. Alternatively, a reducing agent such as dithiothreitol, cysteine, or homocysteine can be added to the extractant to reduce the dehydro form to L-ascorbic acid. Conversely, the L-ascorbic acid can be oxidized to the dehydro form by Norit or enzyme treatment. Total vitamin C assayed as L-dehydroascorbic acid permits use of OPD derivatization with fluorescence detection to quantitate total vitamin C. Metal chelators, usually EDTA, can be added to the extractant to inhibit metal catalyzed oxidation. Use of metaphosphoric acid as the primary component of the extractant has significant metal chelation properties. Any additions to the extractant must be compatible with the resolution and detection modes of the system. Metaphosphoric acid, with or without glacial acetic acid, has been the most common extractant used to extract vitamin C in LC-based methods.

5.3.3.3.2 Chromatography parameters

Supports and mobile phases

Because of the ionic character of ascorbic acid, many different supports can be used. Methods given in Table 5.9 show that reversed-phase systems using C_{18} usually with ion-pairing, or NH_2-bonded supports and macroporous polystyrene divinyl benzene supports are the most commonly applied systems in recently published methods compared to older procedures that relied more on ion-exchange chromatography. Use of NH_2-bonded supports can eliminate the need of ion-pairing reagents. Valls et al[221] used an NH_2-bonded Spherisorb column with potassium phosphate/acetonitrile mobile phase to assay L-ascorbic acid in sausage.

Polymeric styrene divinyl benzene macroporous supports were introduced to food analysis by Lloyd et al.[202,203] The PLRP-S resin has proven to be an advantageous support for LC assay of vitamin C. The support is highly stable to chemical and pH extremes that can degrade ODS supports. Polymeric resins were used to overcome support stability problems encountered in method development studies on the LC analysis of thiamin (Chapter 6).

Mobile-phase selections suitable for vitamin C analysis are as varied as the supports available for resolution. Selection of the PLRP-S resin leads to relatively simple mobile-phase compositions that effectively resolve L-ascorbic acid, L-dehydroascorbic acid, isoascorbic acid, and dehydroisoascorbic acid. The Lloyd et al.[202,203] procedure uses isocratic elution with 0.2 M NaH_2PO_4 at pH 2.14. This system has been used effectively to resolve L-ascorbic acid and isoascorbic acid and their dehydro forms in meat products.[204] Nyyssönen et al.[43] provides an excellent discussion of supports and mobile phase selection for vitamin C analysis.

Detection

Ultraviolet (UV), electrochemical (EC), and fluorescence detection of OPD quinoxaline derivatives of dehydroascorbic acid are the most common detection modes used for quantitation of L-ascorbic acid and its related compounds after LC resolution. Ultraviolet detection of L-ascorbic acid at wavelengths near its absorbance maxima of 245 nm and 265 nm or at 254 nm has frequently been used. Maximal absorbance is dependent on pH. At pH 2.0, it occurs at 245 nm; whereas, at pH 6.4, maximal absorbance is at 265 nm.[43] Ultraviolet absorbance, due to its lack of specificity, is most useful for analysis of high concentration samples such as multivitamins and fruit juices. Absorbance properties of L-dehydroascorbic acid vary considerably from L-ascorbic acid with maximal absorbance occurring around 223 nm at pH 3.1.[25] The low wavelength often leads to erroneous peaks and solvent interference, which complicates the interpretation of the chromatogram.[24] Further, the dehydro form must be present in relatively higher concentrations than L-ascorbic acid because of its weaker

UV absorbance characteristic. Investigators are encouraged to use more specific EC or fluorescence derivative techniques to avoid potential sensitivity and specificity problems inherently characteristic to UV detection. However, as shown in Table 5.9, UV detection is used frequently and with good results.

Brause et al.[224] completed an interlaboratory study of a method developed for organic acid determination in fruit juices (AOAC Official Method 986.13) specifically for ascorbic acid assay. Total ascorbic acid was assayed after reduction of dehydroascorbic acid by dithiothreitol. The reversed-phase system used a simple, isocratic mobile phase of potassium phosphate (0.05% w/v), pH 2.5, containing 0.1% dithiothreitol, and detection at 254 nm. $\%RSD_r$ and $\%RSD_R$ were 5.8–14.7 and 6.4–35.5, respectively. The study involved 19 collaborators in seven countries and seven types of juices. Results were considered good enough to recommend further validation by AOAC International.

At this point in time, LC-MS has not been used as frequently for ascorbic acid analysis as for many of the other water-soluble vitamins. However, Frenich et al.[229] recently showed the power of mass spectrometry to provide identification, confirmation, and quantitation for ascorbic acid when linked to LC. The mass spectrum of ascorbic acid is shown in Figure 5.6.

Summaries of recent LC methods (Table 5.9) indicate that UV and EC detection and use of OPD derivatization for fluorescence detection are the most commonly applied detection modes. EC detection relies upon the oxidation of L-ascorbic acid or isoascorbic acid to the dehydro forms. L-Dehydroascorbic acid is electrochemically inactive. Therefore, EC can be used for total vitamin C only if L-dehydroascorbic acid is reduced to L-ascorbic acid before detection, usually precolumn. A measure of dehydroascorbic acid can be made if total and reduced L-ascorbic acid are independently assayed. The detection limit for EC detection of L-ascorbic acid can be as low as the pmol range. Owing to the improvement in available EC detectors, many researchers are now utilizing the sensitivity and selectivity of EC detection.[200,201,205,207,208,212,217,225]

OPD derivatization is an excellent approach to provide selectivity and sensitivity to vitamin C analysis by LC. Derivatization can be precolumn or postcolumn, since the

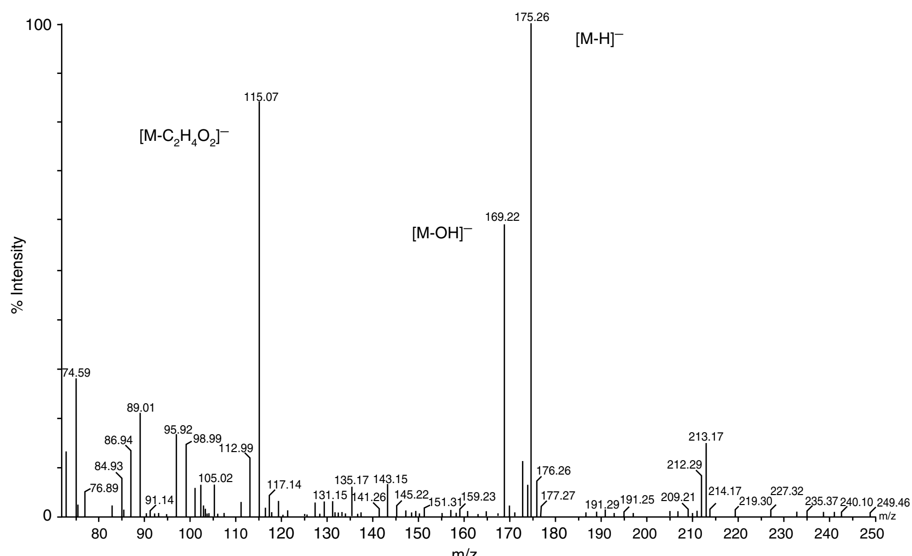


Figure 5.6 Mass spectrum of ascorbic acid. $[M-H]^-$ = unprotonated molecular ion of ascorbic acid, $[M-C_2H_4O_2]^-$ = ascorbic acid fragment. (Reproduced from Frenich, A. G., Torres, M. E. H., Vega, A. B., Vidal, J. L., M., and Bolaños, P. P., *J. Agric. Food Chem.*, 53, 7371, 2005. With permission.)

quinoxaline derivatives can be chromatographically resolved. Well-recognized procedures using OPD derivatization are discussed in the following section.

5.3.3.3.3 LC Methods based on OPD derivatization

Precolumn derivatization

Speek et al.[82] developed a highly useful precolumn procedure using OPD derivatization to assay total vitamin C and total isovitamin C in foods. L-Ascorbic acid and isoascorbic acid and their dehydro forms were extracted with 0.3 M trichloroacetic acid. Ascorbate oxidase was used to enzymatically oxidize L-ascorbic acid and isoascorbic acid to the dehydro forms. OPD derivatization was completed and the quinoxaline derivatives were isocratically resolved on a reversed-phase system (C_{18}) with 0.08 M KH_2PO_4 containing 20% methanol. By omitting the ascorbate oxidase conversion, dehydro forms can be quantitated. This procedure has been the basis of other OPD methods and has been used extensively in other studies.[83,84] The method is provided in Section 5.4.

Procedural steps based upon the AOAC International Microfluorometric Procedure (Method 967.22) were followed to develop an LC procedure with improved sensitivity compared to the manual and semiautomated assays.[206] Complex samples that contained low levels of L-ascorbic acid or produced interferences when assayed by Method 967.22 were successfully assayed by the LC procedure. A clarification step was introduced after the Norit oxidation step that consisted of the addition of sodium acetate and methanol to the Norit-treated extract. OPD derivatives were formed precolumn in the clarified extract. Some high starch samples like potato chips and canned corn required an additional cleanup step to remove solubilized starch before the OPD derivatization. To accomplish the starch removal, an aliquot of the extract was diluted 1:1 with 95% ethanol, incubated in an ice bath, and centrifuged. The chromatography system consisted of a μBondapak C_{18} column and mobile phase of methanol:water (55:45). Fluorescence detection was at Ex $\lambda = 350$ and Em $\lambda = 430$. The AOAC Task Force on Methods for Nutrition Labeling recommended the method for AOAC collaboration.[69] However, the method has not been collaborated. Procedural steps for the method are provided in Section 5.4.

Postcolumn derivatization

Vanderslice and coworkers published a series of papers that refined LC procedures on the basis of postcolumn derivatization with OPD.[196–199] Their final method used an extractant containing 30 g metaphosphoric acid and 0.5 g EDTA dissolved in 500 mL H_2O and 80 mL glacial acetic acid. The solution was diluted to 1 L for extraction of most foods. Animal tissue required an increased HPO_3 concentration to 10% wt/v. The method differs from most vitamin C procedures in that quantitation was based on the use of isoascorbic acid as an internal standard. Chromatography was based on work of Lloyd et al.[202,203] with two PLRP-S columns in series. The basic methodology was used to complete an extensive survey of the vitamin C content in foods in the U.S. diet.[204] Chromatograms are given in Figure 5.7.

5.3.3.3.4 Internal standards. Internal standards have not been used routinely for LC analysis of vitamin C. When derivatives are used to increase sensitivity and selectivity, the internal standard must react similarly to L-ascorbic acid or L-dehydroascorbic acid. Vanderslice and Higgs[197] clearly showed this problem. They examined 23 different compounds for suitability as an internal standard for postcolumn OPD derivatization. The derivatives either coeluted or were not sufficiently soluble in the eluting buffer. Many of the compounds gave no detector response under the elution conditions. Vanderslice and Higgs,[197] however, demonstrated the usefulness of using isoascorbic acid as an internal standard in foods to which it has not been added during processing. Also, for many processed meats, L-ascorbic acid can be used as the internal standard for quantitating isoascorbic acid.

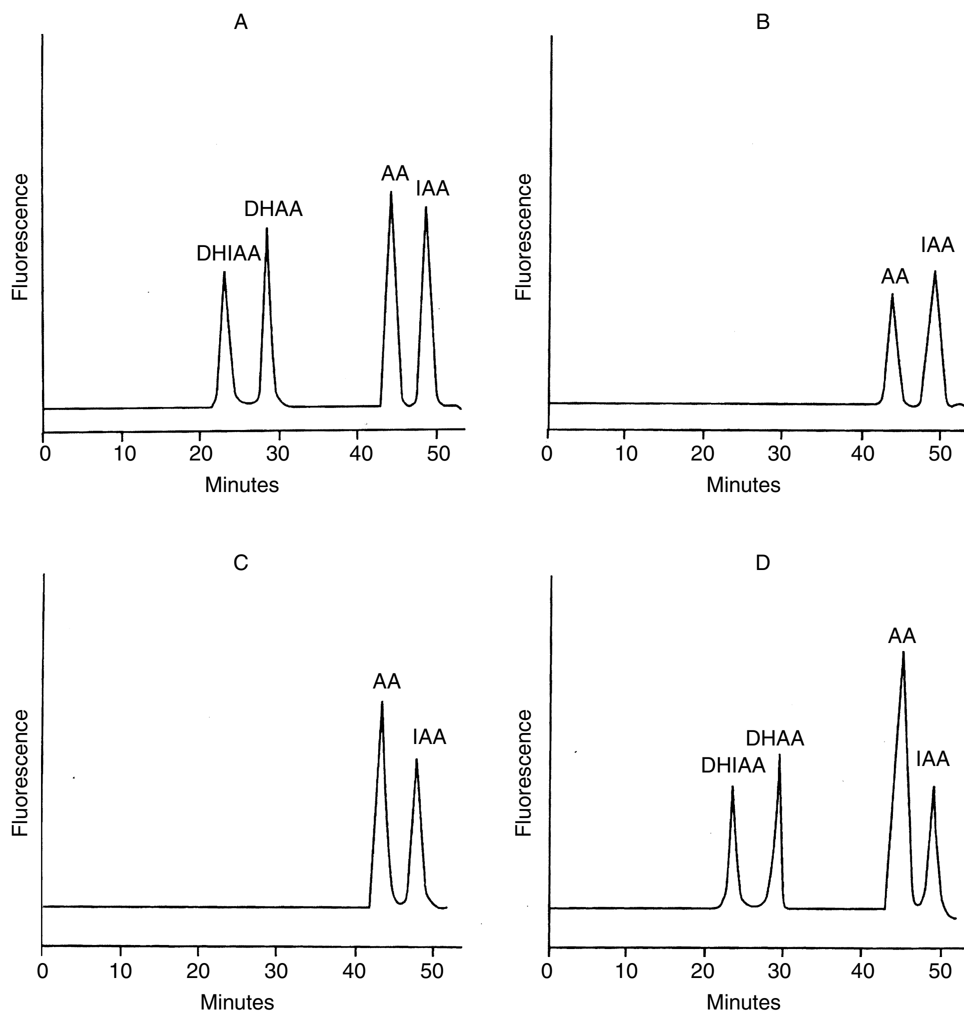


Figure 5.7 Chromatograms of L-ascorbic acid and isoascorbic acid in meat products. Either isoascorbic acid or ascorbic acid was used as IS: (A) Standards, (B) Meat spiked with IAA, (C) Hot dog with AA, (D) Hot dog spiked with AA. (Reproduced from Vanderslice, J. T. and Higgs, D. J., *J. Micronutr. Anal.*, 4, 109, 1988. With permission.)

5.4 Status of vitamin C analysis

The analyst has a wide selection of accurate methods to choose from that have been applied to many biological matrices. LC methods can accurately differentiate between L-ascorbic acid and isoascorbic acid and the dehydro forms of the epimers. However, because of the variability that exists in the application of the methods between laboratories, considerable variation in assayed values can occur when different laboratories assay like samples using proven methods of their choice. This fact was demonstrated by the European Community FLAIR Programme Interlaboratory Assay Method Comparison Study on plasma vitamin C assays.[230] In this study, nine laboratories used DNPH and OPD manual procedures or LC procedures based on OPD derivatization and fluorescence detection, UV, or electrochemical detection. The methods were in-house preferred procedures in routine use at the laboratories conducting the study. Samples included spiked plasma samples of known concentration stabilized by addition of an equal volume of 10% metaphosphoric acid. Results of the

study from two different plasma samples at three concentrations of L-ascorbic acid showed %CV values ranging from 13% to 87%. Greatest CV% variations occurred at low L-ascorbic acid levels of 4.0 and 4.5 mM. Reasons for the variability summarized in the report included the following:

1. Vitamin C is easily oxidized. Plasma samples must be immediately acidified and frozen. Stabilization was performed in different ways by the participating laboratories.
2. A variety of assay procedures are available that differ in fundamental principle.
3. A biological quality control material does not exist with certified L-ascorbic acid levels for use as an in-house method validation tool.
4. Methods are still in use that measure only the reduced or oxidized forms of vitamin C.
5. Some assay methods are subject to positive or negative interferences by other components in the plasma.

Recommendations coming from the study included

1. Development of a procedure-calibration protocol with emphasis on low-level spiking of "real samples" to check recoveries at deficiency or borderline concentration ranges.
2. Use of a quality control sample at the borderline concentrations and at two or three higher levels that are stabilized with 5% metaphosphoric acid and stored at 280°C or lower.
3. An urgent need exists for generally available quality control materials with certified vitamin C contents.

The study presents valuable lessons for all involved in micronutrient analysis programs.

5.5 Method protocols

Rapid Enzymatic Assay for Ascorbic Acid in Various Foods Using Peroxidase
J. Food Sci., 58, 619, 1993[87]

Principle

Ascorbic acid was determined by measuring the change in absorbance during oxidation by quaiacol peroxidase.

Chemicals

- Extraction solution—2% metaphosphoric acid
- EDTA
- 2-mercaptoethanol
- USP ascorbic acid

Apparatus

- Spectrophotometer

Procedure

Extraction

- To quartz cuvette, add 2.6 mL M/30 phosphate buffer, pH 7.0; 150 mL quaiacol peroxidase (0.5 mg mL^{-1} in M/30 phosphate buffer, containing 1.81 mM EDTA and 0.13 mM 2-mercaptoethanol).

- Add 0.3 mL sample.
- Record initial absorbance at 265 nm.
- Initiate reaction with 15 mL of 50 mM H_2O_2.
- Record absorbance after 20 min.
- Calculate ascorbic acid from calibration plot of DA_{265} versus ascorbic acid concentration.
- Calibration curve ranges from 0.2 to 1 mg 100 mL^{-1}.

Flow-Injection Spectrophotometric Determination of Ascorbic Acid in Pharmaceutical Products with the Prussian Blue Reaction

Talanta, 43, 971, 1996[70]

Principle

A deep blue solution forms when Fe^{3+} is reduced to Fe^{2+} by ascorbic acid. The colored complex is monitored at 700 nm.

Chemicals

- Nitric acid
- Fe(III) reagent
- Fe(III) nitrate in 0.014 M nitric acid

$$1.0 \times 10^{-4}, 5.0 \times 10^{-3}, 1.0 \times 10^{-3}, 1.0 \times 10^{-2}\ M$$

- Hexacyanoferrate(III)

$$5.0 \times 10^{-2}, 5.0 \times 10^{-3}\ M$$

- Complexing reagent

0.5% oxalic acid in 0.1 M NaOH

Apparatus

- Eight channel peristaltic pump
- Spectrophotometer with flow-through cell
- Recorder

Procedure

Extraction

- Dissolve samples in 0.014 M nitric acid.

Analysis

- Follow flow-injection procedure schematically shown in Figure 5.8.
- Monitor Prussian Blue at 700 nm.

Determination of Human Plasma and Leukocyte Ascorbic Acid by Microtiter Plate Assay

Nutr. Biochem., 7, 179, 1996[81]

Principle

A microtiter plate technique is used to measure the DNPH derivative of ascorbic acid.

Chemicals

- Ascorbic acid standard 0–2.0 mg 100 mL^{-1}
- Trichloroacetic acid (TCA)

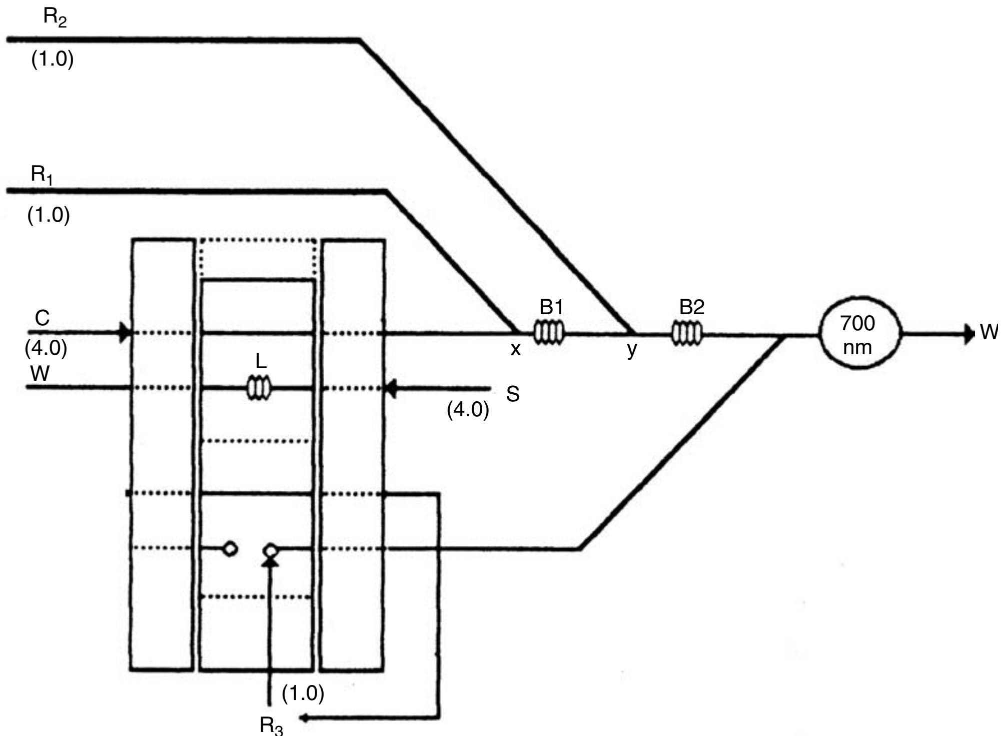


Figure 5.8 Schematic for flow injection analysis of Prussian Blue ascorbic acid analysis. Flow diagram with colorimetric reagents (R_1 and R_2) introduced by confluence (x and y points). B_1 and B_2 are reactors of 150 and 100 cm, respectively. L is a sample loop of 100 cm C, carrier stream; W, waste; S, sample or reference solution. R_3 is an intermittent flow of an alkaline oxalate solution. Numbers between parentheses represent the flow rates. (Reproduced from Nobrega, J. A. and Lopes, G. S., *Talanta*, 43, 971, 1996. With permission.)

- DNPH/thiourea/copper solution (DTC)
- Sulfuric acid

Whole blood fractionation is shown in Figure 5.9.

Apparatus

- 96-well polystyrene microtiter plate
- Microtiter plate reader
- Centrifuge

Procedure

- To 100 mL of TCA-stabilized sample, add 20 mL of DTC solution.
- Incubate at 37°C in dark for 2 and 4 h, vortex at 30 min intervals.
- Place plates on ice.
- Incubate in darkness, 1 h, with vortexing after 30 min.
- Read at 515 nm and 562 nm.

Determination of Total Vitamin C in Various Food Matrices by Liquid Chromatography and Fluorescence Detection

J. AOAC Int., 75, 887, 1992[206]

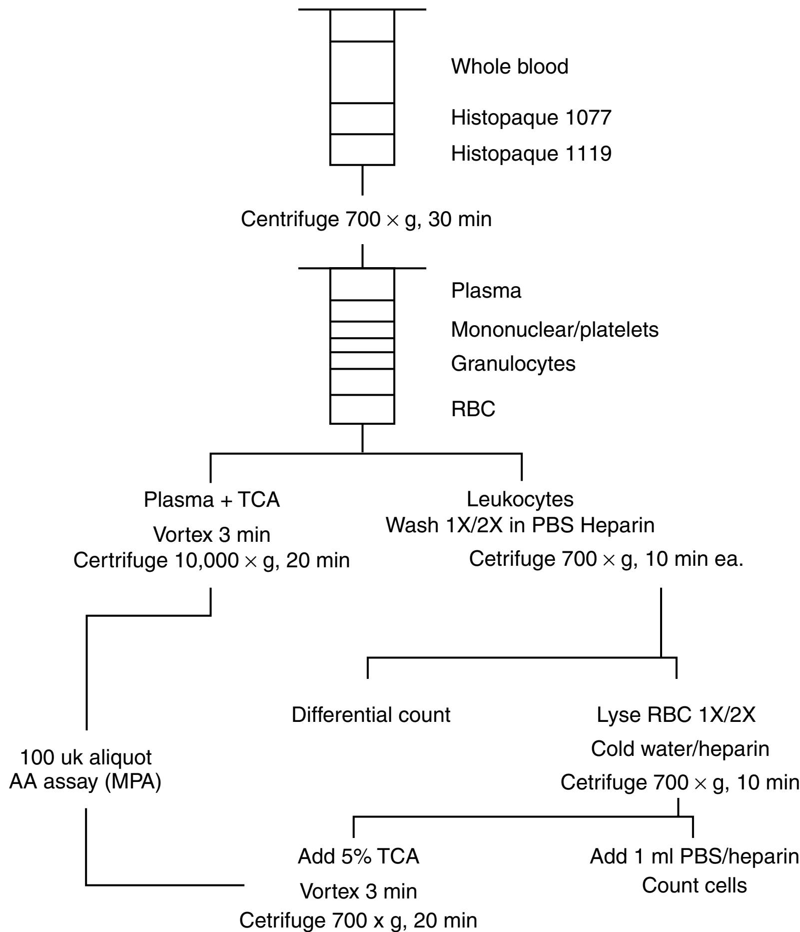


Figure 5.9 Isolation of leukocytes from human blood. (Reproduced from Wei, Y., Ota, R. B., Bowen, H. T., and Omaye, S. T., Determination of human plasma and leukocyte ascorbic acid by microtiter plate assay, *Nutr. Biochem.*, 7, 179, 1996. With permission.)

Principle

Food products were extracted with 3% metaphosphoric acid [(MPA)-acetic acid]. Ascorbic acid was oxidized to dehydroascorbic acid with Norit. *o*-Phenylenediamine fluorescent derivatives were formed precolumn and resolved on μBondapak C_{18}.

Chemicals

- Extraction solution—3% MPA in 8% acetic acid
- USP ascorbic acid
- Sodium acetate
- Acid-washed Norit
- Methanol
- *o*-Phenylenediame (OPD)

Apparatus

- Liquid chromatograph
- Fluorescence detector

Procedure

Extraction

- Homogenize product in food processor.
- Weigh sample portion to contain 1.5–7.5 mg ascorbic acid.
- Mix with 100 mL extraction solution.
- Transfer contents to 250 mL g/s flask containing 2 g Norit.
- Shake, 30 s.
- Filter.
- Transfer 20 mL aliquot (300–700 mg ascorbic acid) to 100 mL volumetric flask containing 5 mL sodium acetate solution (500 g $NaOAC{\cdot}3H_2O$ per L) and 55 mL methanol.
- Dilute to volume with water.
- Filter.
- Transfer aliquot of filtrate to 100 mL volumetric containing 10 mL of OPD solution (2.5 mg mL^{-1}).
- Dilute to volume with mobile phase.

Chromatography

Column	30 cm × 3.9 mm
Stationary phase	μBondapak C_{18}
Mobile phase	methanol:water (55:45)
Column temperature	Ambient
Flow rate	1 mL min^{-1}
Injection	100 μL
Detector	Fluorescence, Ex λ = 350, Em λ = 430
Calculation	External standard, peak area, linear regression

Note: Some samples require clarification of the extract to remove starch. Extracts were clarified by adding an equal volume of 95% ethanol and filtration before the OPD derivatization.

Quantitative Determination of Ascorbic, Dehydroascorbic, Isoascorbic, and Dehydroisoascorbic Acids by LC in Foods and Other Matrices

Assoc. Off. Anal. Chem., 48, 1248, 1965;[48] *J. Micronutr. Anal.* 4, 109, 1988;[197] *J. Food Comp. Anal.* 3, 105, 1990;[198] *J. Nutr. Biochem.*, 4, 184, 1993[199]

Principle

Ascorbic acid (AA) and dehydroascorbic acid (DHAA) are extracted with metaphosphoric acid, resolved, and quantitated by isocratic reversed-phase LC with postcolumn derivatization of DHAA with o-phenylenediamine to give a fluorescent quinoxaline derivative.

Chemicals

- Metaphosphoric acid (MPA)
- Glacial acetic acid
- EDTA (disodium salt)
- *n*-Butanol
- Hexane

Extraction Solution #1 for Foods

- 30 g MPA, 0.5 g EDTA dissolved in 500 mL H_2O and 80 mL glacial acetic acid, and diluted to 1 L

Extraction Solution #2 for Tissue

- 10%, (v/v) MPA
- Standard isoascorbic acid
- DHAA and DHIAA must be prepared and purified using method cited in J

Apparatus

- Waring blender
- Polytron homogenizer
- Centrifuges
- Liquid chromatograph
- Fluorescence detector

Procedure

Extraction of Standards and Samples—Solid Foods

- Before blending sample add 10 mL extraction solution #1.

a. For nonfat and low-starch food
- Transfer 10 mL aliquot to tube and centrifuge at 1200 g at 4°C for 5 min.
- Filter (0.45 mm).
- Inject 100 µL onto LC column.

b. For low-starch foods with fat
- Add 10 mL hexane to 10 mL aliquot.
- Vortex 1 min.
- Centrifuge at 1200 g.
- Filter (0.45 mm) aqueous layer.
- Inject 100 µL onto LC column.

c. For high starch foods
- Add 10 mL *n*-butanol to 10 mL aliquot.
- Vortex 1 min.
- Centrifuge 48,400 g at 4°C for 10 min.
- Filter (0.45 mm) aqueous layer.
- Inject 100 µL onto LC column.

Chromatography

Column	15.0 cm × 4.6 mm and 25.0 cm × 4.6 mm
Stationary phase	PLRP-S
Mobile phase	0.2 M NaH_2PO_4 (adjusted to pH 2.14 with H_3PO_4)
Column temperature	Ambient
Flow rate	0.8 mL min^{-1}
Injection	100 µL
Postcolumn oxidation and fluorescent derivatization	Oxidant stream = 0.5 mM L^{-1} $CuCl_2$ Reactant stream = 3.1 mM L^{-1} *o*-phenylenediamine Reaction coil temperature = 70°C Cooling coil temperature = 20°C

Detection	Fluorescence, Ex λ = 350, Em λ = 430
Calculation	Peak area, external standard if both AA and IAA are present

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chapter six

Thiamin

6.1 Review

Thiamin was isolated and characterized in 1926. It was the first of the water-soluble vitamins structurally characterized and was formally given the designation vitamin B_1 in 1927 by the British Medical Research Council. Descriptions of beriberi, the principal form of thiamin deficiency, were recorded centuries earlier, and associations of the deficiency to diets high in polished rice were made before the twentieth century. Beriberi was epidemic in populations consuming polished rice as a dietary staple until rice fortification became a common practice. Thiamin deficiency occurs as a marginal deficiency and in the more extreme states of beriberi and Wernicke–Korsakoff's syndrome. Marginal deficiency is characterized by fatigue, irritability, and lack of concentration. Beriberi, resulting from prolonged, low dietary intake, includes dry beriberi (muscle wasting with heart involvement, hypotension, sodium retention, tachycardia, and pulmonary edema), and wet beriberi (edema, anorexia, muscle weakness, ataxia, and peripheral paralysis). Infantile beriberi, which can be quickly fatal, is characterized by vomiting, convulsions, abdominal distention, and anorexia. Infantile beriberi can result in heart failure.[1–3] Wernicke–Korsakoff syndrome is a severe deficiency state characterized by mental disorder, including confusion, hallucinosis, psychosis, and coma. It is frequently seen in alcoholics after long periods of alcohol intake without food intake. Clinically, thiamin status is indicated by measurement of urinary excretion of thiamin and erythrocyte transketolase activity. Transketolase requires thiamin pyrophosphate (TPP) and is a highly reliable measure of thiamin status. Serum, erythrocyte, and blood thiamin levels are insensitive thiamin status indices.[2,4,5] Measurement of erythrocyte transketolase as a thiamin index requires measurement of the basal activity, which is dependent upon the TPP levels before stimulation with added TPP, measurement of the stimulated level after TPP addition, and then, calculation of the activity coefficient (AC).[4]

$$AC = \frac{\text{Enzyme activity (with TPP)}}{\text{Basal enzyme activity (without TPP)}}$$

If status is adequate, added TPP has minimal effect on the AC. If status is inadequate, the AC increases. Marginal deficiency is indicated at AC levels of 1.20–1.25.[2] Inadequate intake of thiamin, problems with absorption and transport, and decrease in TPP synthesis, all can lead to deficiency.[6] In developed countries, severe thiamin deficiency is rare; however, marginal deficiency is more frequently documented.

Dietary sources considered to be primary sources to the human include fortified breakfast cereals, unrefined cereal grains, enriched cereal grain products, legumes, nuts, and pork, which is higher in thiamin content than most other muscle foods. Table 6.1 shows the

Table 6.1 Thiamin Content of Various Foods

Description	NDB No.	mg 100 g^{-1}	Description	NDB No.	mg 100 g^{-1}
Cereals ready-to-eat, GENERAL MILLS, Whole Grain TOTAL	08077	7.03	Wheat flour, white, bread, enriched	20083	0.81
Cereals ready-to-eat, KELLOGG, KELLOGG'S Complete Wheat Bran Flakes	08028	5.40	Wheat flour, white, all-purpose, enriched, bleached	20081	0.78
Cereals ready-to-eat, KELLOGG, KELLOGG'S PRODUCT 19	08058	5.00	Cornmeal, degermed, enriched, yellow	20022	0.72
Cereals ready-to-eat, GENERAL MILLS, TOTAL Corn Flakes	08246	5.00	Pork, fresh, leg (ham), whole, separable lean only, cooked, roasted	10011	0.69
Cereals ready-to-eat, GENERAL MILLS, TOTAL Raisin Bran	08247	2.73	Pork, cured, ham, whole, separable lean only, roasted	10153	0.68
Cereals ready-to-eat, KELLOGG, KELLOGG'S RICE KRISPIES	08065	2.64	Cornmeal, self-rising, degermed, enriched, yellow	20025	0.68
Cereals ready-to-eat, GENERAL MILLS, FROSTED WHEATIES	08266	2.50	Rice, white, long-grain, parboiled, enriched, dry	20046	0.60
Cereals ready-to-eat, GENERAL MILLS, WHEATIES	08089	2.50	Pork, fresh, shoulder, arm picnic, separable lean only, cooked, braised	10078	0.60
Cereals ready-to-eat, QUAKER, CAP'N CRUNCH'S PEANUT BUTTER CRUNCH	08012	2.33	Rice, white, long-grain, regular, raw, enriched	20044	0.58
Cereals ready-to-eat, KELLOGG, KELLOGG'S ALL-BRAN Original	08001	2.27	Fish, tuna, yellowfin, fresh, cooked, dry heat	15221	0.50
Cereals ready-to-eat, KELLOGG, KELLOGG'S FROOT LOOPS	08030	2.25	Wheat flour, whole-grain	20080	0.45
Cereals ready-to-eat, KELLOGG, KELLOGG'S Corn Flakes	08020	2.13	Snacks, trail mix, tropical	19061	0.45
Cereals ready-to-eat, KELLOGG, KELLOGG'S CRISPIX	08259	1.90	Buckwheat flour, whole-groat	20011	0.42
Cereals ready-to-eat, KELLOGG, KELLOGG'S SPECIAL K	08067	1.68	Cornmeal, whole-grain, yellow	20020	0.39
Cereals ready-to-eat, QUAKER, CAP'N CRUNCH	08010	1.58	Cereals, CREAM OF WHEAT, mix'n eat, plain, prepared with water	08109	0.30
Pork, fresh, loin, center loin (chops), bone-in, separable lean only, cooked, pan-fried	10176	1.24	Breakfast items, biscuit with egg and sausage	21005	0.28
Oat bran, raw	20033	1.17	Orange juice, frozen concentrate, unsweetened, undiluted	09214	0.28
Pork, fresh, loin, center loin (chops), bone-in, separable lean only, cooked, broiled	10042	1.15	Soybeans, green, cooked, boiled, drained, without salt	11451	0.26
Pork, cured, ham, extra lean and regular, canned, roasted	10185	0.96	Noodles, egg, spinach, cooked, enriched	20112	0.25
Ham, sliced, extra lean	07028	0.93	Rice, white, long-grain, parboiled, enriched, cooked	20047	0.25

Source: Data from United States Department of Agriculture, Agricultural Research Science, 2006, USDA Nutrient Database for Standard Reference, Release 19, Nutrient Data Laboratory Home Page, http://www.nal.gov/finic/foodcomp, Riverdale, MD. Nutrient Data Laboratory, USDA.

ranking of some of the more significant dietary sources for thiamin as presented in the USDA National Nutrient Database for Standard Reference, Release 18.[7] This ranking is based on thiamin content (mg 100 g^{-1}). Fortified breakfast cereals contain >2 times the amount of thiamin present in pork products, which are considered the most concentrated nonenriched or nonfortified food source for thiamin.

Recommended Dietary Allowances (RDA) as set by the Institute of Medicine in the Dietary Reference Intake (DRI) for thiamin are 1.2 and 1.1 mg d^{-1} for adult men and women, respectively (Table 6.2).[2] For pregnancy and lactation, the RDA increases to 1.4 mg d^{-1}. The median thiamin intake in the United States is estimated to be 2 mg d^{-1} for young men and 1.2 mg d^{-1} for young women.[2] Fewer than 5% of the population, except for lactating females, had intakes less than the Estimated Average Requirements of 1.0 mg d^{-1} for men and 0.9 mg d^{-1} for women. The Nutritional Labeling and Education Act of 1990 (NLEA) specifies a Reference Daily Intake (RDI) of 1.5 mg for use in nutritional labeling.[8]

Thiamin exists naturally as free thiamin and phosphorylated as thiamin monophosphate (TMP), thiamin diphosphate or TPP, and thiamin triphosphate (TTP). All forms exist in animal and plant tissue, although plant tissue contains higher levels of the free vitamin

Table 6.2 Dietary Reference Intakes for Thiamin

Life stage	DRI (mg d^{-1})
Infants (months)	
0–6	0.2
7–12	0.3
Children (years)	
1–3	**0.5**
4–8	**0.6**
Males (years)	
9–13	**0.9**
14–18	**1.2**
19–30	**1.2**
31–50	**1.2**
51–70	**1.2**
>70	**1.2**
Females (years)	
9–13	**0.9**
14–18	**1.0**
19–30	**1.1**
31–50	**1.1**
51–70	**1.1**
>70	**1.1**
Pregnancy (years)	
≤ 18	**1.4**
19–30	**1.4**
31–50	**1.4**
Lactation (years)	
≤ 18	**1.4**
19–30	**1.4**
31–50	**1.4**

Bold type: recommended dietary allowance; Ordinary type: adequate intake (AI).

Source: Food and Nutrition Board, Institute of Medicine, *Dietary Reference Intake for Thiamin, Riboflavin, Niacin, Vitamin B_6, Folate, Vitamin B_{12}, Pantothenic Acid, Biotin, and Choline*, National Academy of Sciences Press, Washington, DC, 2000, Chap. 4

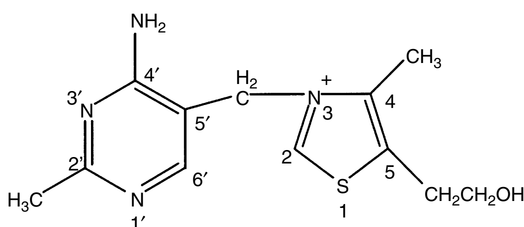
than found in animal tissue. TPP is one of the principle cofactors for decarboxylation of α-keto acids characterized by the role of pyruvate dehydrogenase complex in synthesis of acetyl-CoA from pyruvate in the trichloroacetic acid (TCA) cycle. TPP also functions as the cofactor for the transketolase reaction in the pentose phosphate pathway. The transketolase reaction transfers a two-carbon fragment from xylulose-5-phosphate to ribose-5-phosphate, forming glyceraldehyde-3-phosphate and the seven-carbon sugar, sedoheptulose-7-phosphate. TPP plays a central role in metabolism and is essential to normal carbohydrate, nucleic acid, and amino acid metabolism. TPP and other thiamin phosphorylated esters function in nerve impulse transmission. The exact role of thiamin in neurotransmission is unknown; however, TPP is involved in synthesis of acetylcholine, a primary neurotransmitter. More recently, TPP was shown to be essential in metabolism of *n*-methyl-branched fatty acids.[9] TPP and TTP are both significant to brain cell function.[10] TPP-dependent enzymatic reactions were reviewed by Jordan.[11]

6.2 Properties

6.2.1 Chemistry

6.2.1.1 General properties

The structure of thiamin in the free base form is given in Figure 6.1. The vitamin is characterized by a pyrimidine ring (4′-amino-2′-methylpyrimidinyl-5′-ylmethyl) linked by a methylene bridge to the 3-nitrogen atom in a substituted thiazole (5-(2-hydroxyethyl)-4-methylthiazole). Conversion of thiamin to TMP, TPP, and TTP is shown in Figure 6.2. Thiamin hydrochloride ($C_{12}H_{18}ON_4SCl_2$) and thiamin mononitrate ($C_{12}H_{17}O_4N_5S$) are the commercially available forms used in pharmaceuticals and for food fortification (Figure 6.2). Thiamin hydrochloride is the United States Pharmacopeial Convention (USP) reference standard. Physical properties of the thiamin salt and naturally occurring phosphorylated forms are given in Table 6.3. Thiamin hydrochloride is a white crystalline powder with a yeast-like odor and salty, nut-like taste.[14] The most distinguishing difference between the hydrochloride salt and the mononitrate is water solubility. The hydrochloride is soluble in water (1 g mL^{-1}) and the mononitrate is only slightly water soluble (0.027 g mL^{-1}). This solubility difference leads to differentiation in industrial uses for the two thiamin forms. The hydrochloride salt is used in injectable and parenteral pharmaceuticals and for fortification of foods requiring solubility. The lower hydroscopicity of the mononitrate makes it ideal for use in dry blends, multivitamins, and dry products such as enriched flour.[15] Thiamin hydrochloride is nearly insoluble in methanol, ethanol, and glycerol. It is insoluble in ether, acetone, benzene, hexane, and chloroform.



Thiamin (free-base)
3-((4′-Amino-2′-methyl-5′pyrimidinyl)methyl)-
5-(2-hydroxyethyl)-4-methylthiazole
Vitamin B_1

Figure 6.1 Structure of thiamin.

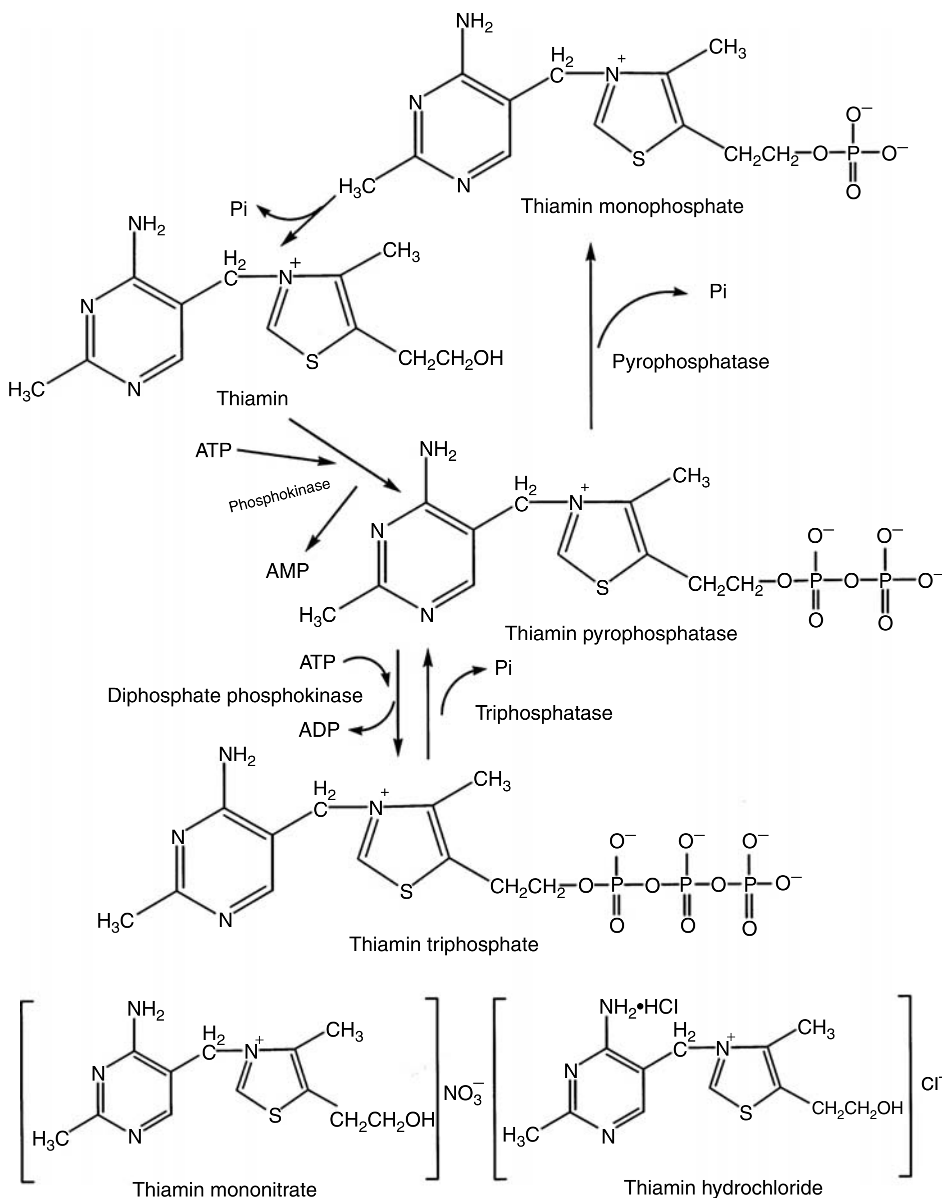


Figure 6.2 Structures of thiamin and related compounds.

6.2.1.2 Spectral properties

Thiamin hydrochloride absorbs in the region of 200–300 nm. Absorption maxima vary with the pH of the solution. At pH 2.9, a single maxima is present at 246 nm. At pH levels above 5 but below neutrality, two absorption maxima exist at 234 nm and 264 nm, representing the pyrimidine and thiazole rings, respectively.[12] The $E^{1\%}_{1\mathrm{cm}}$ values in phosphate buffer at 246 nm (pH 2.9), 234 nm (pH 5.5), and 264 nm (pH 5.5) are 425, 345, and 255, respectively (Table 6.3).

Oxidation of thiamin or its phosphate esters with alkaline potassium ferricyanide or thiamin-bromide quantitatively forms thiochrome (Thc), thiochrome monophosphate (ThcMP), thiochrome pyrophosphate (ThcPP), and thiochrome triphosphate (ThcTP) (Figure 6.3). At alkaline pH above 8.0, thiochrome products have excitation maxima at 375 nm and emission maxima at 432–435 nm (strong blue fluorescence).[16,17] These highly fluorescent

Table 6.3 Physical Properties of Thiamin Salts

Substance[a]	Molar mass	Formula	Solubility	Crystal form	Melting point°C	Absorbance[b]		
						λ max (nm)	$E^{1\%}_{1cm}$	ε × 10^{-3}
Thiamin hydrochloride CAS No. 67-03-5 **9430**	337.26	$C_{12}H_{17}ClN_4OS \cdot HCl$	Water—1.0 g mL^{-1} Ethanol (95%) 1.0 g 100 mL^{-1} Glycerol—5 g 100 mL^{-1} Insol. in ether, benzene, hexane, chloroform Water—2.7 g 100 mL^{-1}	White crystalline powder, colorless, monoclinic, needles	246–250 (dec.)	246[c] 234[d] 264[d]	425 345 255	[14.3] [11.6] [8.6]
Thiamin mononitrate CAS No. 532-43-4 **9430**	327.37	$C_{12}H_{17}N_5O_4S$	Water—2.7 g 100 mL^{-1}	White to yellow, white crystals	196–200 (dec.)			
Thiamin monophosphate CAS No. 532-40-1 **9431**	344.33	$C_{12}H_{17}N_4O_4PS$						
Thiamin pyrophosphate CAS No. 157-87-0 **9431**	424.31	$C_{12}H_{18}N_4O_7P_2S$			(Tetrahydrate form) 220–222 (dec.)			
Thiamin triphosphate	504.29	$C_{12}H_{19}N_4O_{10}P_3S$			(Hydrochloride form) 228–232 (dec.)			

[a] Common or generic name; CAS No.: 2 Chemical Abstract Service number, bold print designates the Merck Index monograph number.
[b] Values in brackets are calculated from corresponding $E^{1\%}_{1cm}$ values.
[c] In 0.1 M phosphate buffer, pH 2.9.
[d] In 0.1 M phosphate buffer, pH 5.5.

Sources: Ball, G. F. M., Chemical and biological nature of the water-soluble vitamins, In *Water-soluble Vitamin Assays in Human Nutrition*, Chapman and Hall, New York, 1994, Chap. 2; Budavari, S., *The Merck Index*, 13th ed., Merck and Company, Whitehouse Station, NJ, 2001, p. 1586.; Ellefson, W. C., *Thiamin, Methods of Vitamin Assay*, 4th ed., Augustin, J., Klein, B. P., Becker, D. A., and Venugopal, P. B., eds., John Wiley and Sons, New York, 1985, Chap. 13.

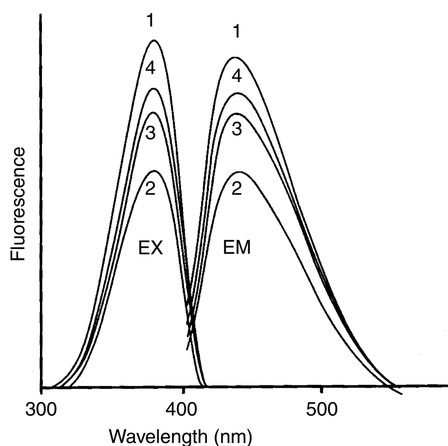


Figure 6.3 Fluorescence excitation and emission spectra of Thc (1), ThcMP (2), ThcPP (3), and ThcTP (4). (Reproduced from Ishii, K., Sarai, K., Sanemori, H., and Kawasaki, T., *Anal. Biochem.*, 97, 191, 1979. With permission.)

oxidation products are the basis of the thiochrome procedure for thiamin quantitation.[14] Thiochrome analysis has been used to develop most of the analytical data available on the thiamin content of the food supply.[18] Since thiamin does not fluoresce, development of the thiochrome analysis in the 1930s provided a highly specific method for thiamin quantitation from most matrices.

6.2.2 Stability

Thiamin is one of the least stable of the water-soluble vitamins when the pH of the matrix approaches neutrality. Maximum stability in solution is between pH 2.0 and 4.0. Therefore, in low-acid foods, the vitamin is highly susceptible to losses during thermal processing.[18] Thiamin is highly unstable at alkaline pH. Stability is dependent upon the extent of heating and on the food matrix characteristics. Thermal degradation occurs even under slightly acid conditions. Dwivedi and Arnold[19] found that thermal degradation was primarily due to scission of the methylene bridge yielding pyrimidine and thiazole. Further destruction of thiazole liberates hydrogen sulfide. Both reactions are influenced by oxidation–reduction, inorganic bases (sulfites and bisulfites), metal complexes, radiation, and thiaminases with pH being the controlling factor. At pH 6.0 or greater, sulfite treatment cleaves the methylene bridge with formation of 2-methyl-4-amino-5-methylprimidylsulfonate and 4-methyl-5-(2-hydroxyethyl)thiazole.[6] Jhoo et al.[20] reported nucleophilic substitution to the methylene bridge carbon by 2-methyl-4-amino-5-(2-methyl-3-furylthiomethyl)pyrimidine. This was the first reported nucleophilic substitution at the methylene bridge of thiamin. Substitution occurred at pH 6.5 at 110°C. Ball[12] reported that alkaline pH during cooking or processing leads to extensive thiamin losses. Use of baking powder in cake mixes was mentioned as an example where 50% or higher loss can occur. Thiamin is destroyed in baked chocolate products.

Various recent studies are available that define thiamin stability during processing and storage of foods.[21–29] Thiamin is known to be the most radiation-sensitive water-soluble vitamin. Studies by van Calenberg et al.[21] showed 31% and 28% loss in chicken meat irradiated with x-rays (50 Gy min^{-1}) and electrons (5 kGy min^{-1}), respectively, to a dose of 3-kGy. Stability of thiamin is affected to a greater extent by glass transition compared to water activity (a_w). Bell and White[22] concluded from their studies that thiamin stability in a food will be greater if low-moisture products are formulated to have as high a glass transition temperature (T_g) value as possible.

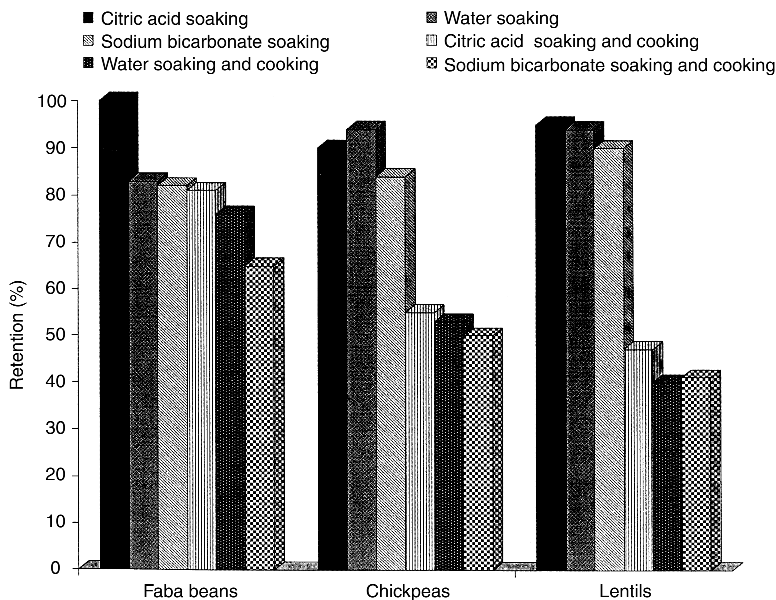


Figure 6.4 Effect of processing on the thiamin content of legumes. (Reproduced from Prodanov, M., Sierra, I., and Vidal-Valverde, C., *Food Chem.*, 84, 271, 2004. With permission.)

Soaking and cooking of legumes can have a pronounced effect on thiamin levels. Prodanov et al.[24] state that as much as 51% of the thiamin in legumes can be lost through such processes (Figure 6.4). Baking of whole wheat bread led to 48% loss of thiamin.[26] Germination and fermentation of seeds can increase water-soluble vitamin content. Thiamin was shown to increase during germination of lentils but not through natural fermentation.[27–29]

Thiaminase is present in unprocessed foods of plant and animal origin. Thiamin degradation occurs either by nucleophilic displacement of the methylene of the pyrimidine or by hydrolysis of thiamin into thiazole and pyrimidine.[19] Thiaminase reactions are initiated by bruising, blending, or homogenization, or other processes that break tissue structure. Sulfhydryl groups and other reducing agents usually protect thiamin from thiaminase action. Degradation of thiamin in the presence of sulfite proceeds through cleavage of the methylene bridge similar to degradation at alkaline pH. Use of sulfite as a food processing aid to inhibit browning reactions can lead to extensive losses.

6.3 Methods

6.3.1 Classical approaches to analysis of thiamin

The following section provides pertinent information on each of these approaches. Reviews available on thiamin assay procedures include Ellfeson,[14] Kawasaki and Egi,[30] Ottaway,[15] Eitenmiller and Landen,[18] and Fayol.[31] Many of the standard procedures are available in various handbooks. These methods are summarized in Table 6.4.

6.3.1.1 Chemical

The thiochrome reaction (Figure 6.5) has been used since its development in 1935[49] for analysis of thiamin from biological matrices. In the absence of other fluorescent compounds that can interfere with the assay, the fluorescence intensity of thiochrome is proportional to the total thiamin in the sample.[14] Thiochrome is used as the determinative step in United States

Table 6.4 Regulatory and Handbook Methods for the Analysis of Thiamin

Source	Form	Methods and application	Approach	Most current cross-reference
***U.S. Pharmacopeia National Formulary*, 2006, USP 29/NF 24, Dietary Supplements Official Monographs**[32]				
1. Pages 2393, 2394, 2396, 2399, 2404–2405, 2420–2421	Thiamin Thiamin hydrochloride	Thiamin in oil- and water-soluble vitamin capsules/tablets, oral solution w/wo minerals	Method 1—LC 280 nm Method 2—LC 254 nm Method 3—LC 270 nm	None
2. Pages 2428–2430, 2432–2433, 2435–2436	Thiamin Thiamin hydrochloride	Thiamin in water-soluble vitamin capsules/tablets w/wo minerals	Method 1—LC 280 nm Method 2—LC 254 nm Method 3—LC 270 nm	None
3. Pages 2121–2122	Thiamin Thiamin hydrochloride	Thiamin hydrochloride (NLT 98.0%, NMT 102.0%)	LC 254 nm	None
4. Pages 2122–2123	Thiamin Thiamin hydrochloride	Thiamin hydrochloride injection/oral solution	LC 254 nm	None
5. Page 2123	Thiamin Thiamin hydrochloride	Thiamin hydrochloride tablets	Thiochrome Fluorescence Ex $\lambda = 365$ Em $\lambda = 435$	None
6. Pages 2123–2124	Thiamin mononitrate	Thiamin mononitrate (NLT 98.0%, NMT 102.0%)	LC 254 nm	None
7. Page 2124	Thiamin mononitrate	Thiamin mononitrate oral solution	LC 254 nm	None
***British Pharmcopoeia*, 2007**[33]				
1. Pages 2026–2027	Thiamin hydrochloride	Thiamin hydrochloride	Potentiometric	None
2. Pages 2028–2029	Thiamin mononitrate	Thiamin nitrate	Titration	None
3. Pages 2955–2956	Thiamin hydrochloride	Thiamin injection	LC 244 nm	None
4. page 2956	Thiamin hydrochloride	Thiamin tablets	LC 244 nm	None
5. Pages 2989–2990	Thiamin hydrochloride	Vitamins B and C injection	LC 280 nm	None
***AOAC Official Methods of Analysis*, 18th ed., 2005**[34]				
1. 45.1.05	Thiamin Thiamin hydrochloride	AOAC Official Method 942.23, Thiamin (Vitamin B_1) in Foods	Thiochrome fluorescence Ex $\lambda = 365$ Em $\lambda = 435$	*J. Assoc. Off. Anal. Chem.*, 64, 616, 1981[52]

Continued

Table 6.4 (Continued)

Source	Form	Methods and application	Approach	Most current cross-reference
***AOAC Official Methods of Analysis*, 18th ed., 2005**[34] (*Continued*)				
2. 45.1.06	Thiamin Thiamin hydrochloride	AOAC Official Method 953.17, Thiamin (Vitamin B_1) in Grain Products	Thiochrome fluorescence Ex λ = 365 Em λ = 435	*J. Assoc. Off. Anal. Chem.*, 36, 837, 1953;[35] 37, 122, 1954;[36] 37, 154, 1954;[37] 38, 722, 1955[38]
3. 45.1.07	Thiamin Thiamin hydrochloride	AOAC Official Method 957.17, Thiamin (Vitamin B_1) in Bread	Thiochrome fluorescence Ex λ = 365 Em λ = 435	*J. Assoc. Off. Anal. Chem.*, 40, 843, 1957;[39] 41, 603, 1958;[40] 43, 47, 1960[41]
4. 50.1.08	Thiamin Thiamin hydrochloride	AOAC Official Method 986.27, Thiamin (Vitamin B_1) in Milk-Based Infant Formula	Thiochrome fluorescence Ex λ = 365 Em λ = 435	*J. Assoc. Off. Anal. Chem.*, 69, 777, 1986[42]
***European Committee for Standardization*, 2003**[46]				
EN14122	Thiamin	Foods	LC Pre- or post-column derivatization to thiochrome Fluorescence Ex λ = 366 Em λ = 420	*J. Micronutr. Anal.*, 5, 269, 1989;[45] *Food Chem.*, 56, 81, 1996;[47] *Fres. J. Anal. Chem.*, 343, 155, 1992[48]
***American Association of Cereal Chemists*, 10th ed., 2000, Approved Methods, vol. 2**[43]				
1. AACC 86-80	Thiamin Thiamin hydrochloride	Thiamin in complete feeds (>0.4 mg/kg)	Thiochrome fluorescence Ex λ = 370 Em λ = 430	*AOAC Official Methods of Analysis*, 18th ed., 2005[34]
2. AACC 86-90	Thiamin Pyridoxine Riboflavin Nicotinic acid Nicotinamide	Thiamin in concentrates	LC 285 nm	None
***Food Chemicals Codex*, 5th ed., 2004**[44]				
1. Pages 411-412	Thiamin hydrochloride	Thiamin hydrochloride (NLT 98.0%, NMT 102.0%)	LC 254 nm	None
2. Page 412	Thiamin mononitrate	Thiamin mononitrate (NLT 98.0%, NMT 102.0%)	LC 254 nm	None

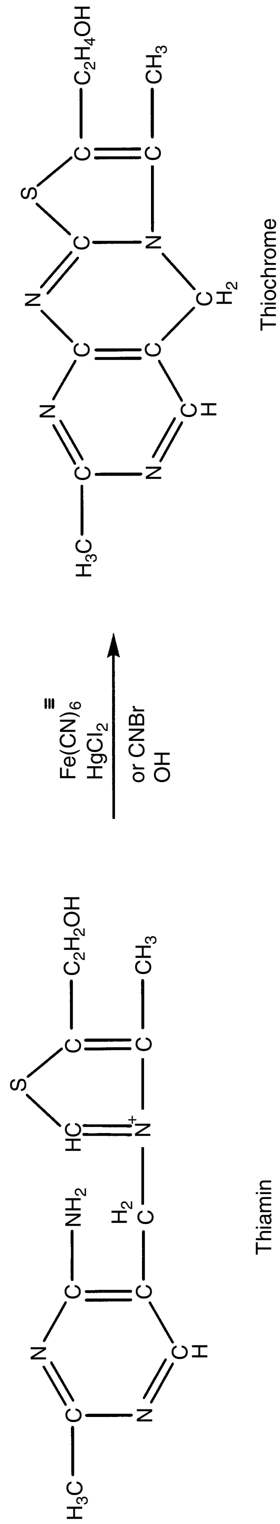


Figure 6.5 Thiochrome reaction.

Pharmacopeia (USP) methods for pharmaceuticals,[32] Association of Official Analytical Chemists (AOAC) International Official Methods,[34] American Association of Cereal Chemists Approved Methods,[43] and Food Chemicals Codex.[44] Procedural descriptions of AOAC International Methods, because of their worldwide influence, are given as follows.

AOAC Official Method 942.23, Thiamin (Vitamin B_1) in Foods, Fluorometric Method, AOAC Official Methods of Analysis, 45.1.05

The method includes the following steps:

1. *Acid Hydrolysis:* Samples are hydrolyzed with 0.1 N HCl for 30 min at 95°C–100°C or autoclaved at 121°C for 30 min. Most thiamin procedures use HCl hydrolysis because the vitamin is stable under the acidic environment. The hydrolysis breaks protein complexes and effectively liberates thiamin from cellular material in most sample matrices.
2. *Enzyme Hydrolysis:* The acid hydrolysate is adjusted to pH 4.0–4.5 with 2 N sodium acetate. Phosphorylated thiamin esters are converted to free thiamin using takadiastase, Mylase 100 (U.S. Biochemical Corporation), or α-amylase. The digest is incubated at 45°C–50°C for 3 h.
   The products of the phosphate esters are insoluble in isobutanol used in the subsequent extraction step and complete conversion must be assured.[14] Proper selection of the phosphatase source, therefore, is a significant but often slighted part of the procedure. Studies by Hasselmann et al[45] found that a mixture of β-amylase and takadiastase was satisfactory for thiamin hydrolysis. β-Amylase, alone, did not produce complete dephosphorylation. Suitability of the enzyme for hydrolysis of thiamin phosphate esters can be determined by digesting a thiamin phosphate standard and comparing the digest to a thiamin reference standard.[14] A 3 h incubation period is considered sufficient for total hydrolysis when the enzyme has high, documented phosphatase activity.[50] Various combined enzyme extractions suitable for the simultaneous release of thiamin with other water-soluble vitamins are discussed in Chapter 14.
3. *Extract Cleanup:* The digest is adjusted to pH 3.5, filtered, and an aliquot containing approximately 5 mg thiamin is passed through an absorption column filled with a 100 mm bed of Bio-Rex 70 (Hydrogen form) (Bio Rad Laboratories) to remove possible interfering compounds. Decalso was formally used as the support, but became unavailable in the 1970s. Bio-Rex 70 was chosen as the replacement. Reversed-phase C_{18} open-column chromatography was reported to be equivalent to Bio-Rex 70 for extract cleanup.[51] The C_{18} cleanup requires elution with cold 3% potassium chloride: methanol (70:30), which is easier to work with than the boiling acid-KCl solution required for elution from Bio-Rex 70.
4. *Thiochrome Formation:* An aliquot of the purified extract is oxidized with 1% potassium ferricyanide in 15% sodium hydroxide. Thiochrome is extracted with isobutanol and quantitated fluorometrically. The blank in the manual thiochrome procedure is produced by eliminating the oxidation step. This blank does not account for fluorescence developed during the oxidation.[52] Several studies have been completed to improve the blank determination. Leveille[53] used benzenesulfonyl chloride (BSC) to inhibit thiochrome formation in the presence of potassium ferricyanide. His procedure for urine analysis eliminated the column chromatography and isobutanol extraction by use of a BSC-treated sample to correct for blank fluorescence. Soliman[52] adapted the BSC technique to food analysis using a semiautomated technique. The method has been used by the Atlanta Center for Nutrient Analysis, Food and Drug Administration (FDA), for a large variety of sample matrices.

AOAC Official Method 953.17, Thiamin (Vitamin B_1) in Grain Products, Fluorometric (Rapid Method), AOAC Official Methods of Analysis, 45.1.06

Method 953.17 is used for enriched products where natural ester forms of thiamin make up a negligible amount of the total thiamin. It is only applicable to enriched grain products since the enzyme digestion and Bio-Rex 70 purification steps are eliminated. The method should not be used until the amount of phosphorylated forms are verified to be negligible when compared to the amount of thiamin hydrochloride added through enrichment.[18] For flour, a nonenriched product can be used for comparative assays.

AOAC Official Method 957.17, Thiamin (Vitamin B_1) in Bread, Fluorometric Method, AOAC Official Methods of Analysis, 45.1.07

Method 957.17 was originally designated for the analysis of air-dried bread. All steps are essentially the same as Method 957.17. The AOAC Task Force on Methods for Nutrition Labeling recommended the method for assay of beverages, juices, fruits, dairy products, fish, and shellfish.[54] The AOAC Official Methods of Analysis, 18th ed. (2005)[34] states that the method is applicable to all foods. Moore and Dolan[55] recently optimized the oxidation steps applicable to AOAC Method 957.17 and AACC Method 86-80 for the thiochrome determination of thiamin in grain products. Their work, which was specific for soft wheat flour, indicated that a range existed between 4.84 and 100 μg of $K_3Fe(CN)_6$ per μg of thiamin hydrochloride for optimal oxidation and fluorescence response. The recommended range for the amount of $K_3Fe(CN)_6$ is lower than recommended in the approved methods.

AOAC Official Method 986.27, Thiamin (Vitamin B_1) in Milk-Based Infant Formula, Fluorometric Method, AOAC Official Methods of Analysis, 50.1.08

Method 986.27 is identical to Method 942.23. The AOAC Task Force on Methods for Nutrient Labeling recommended the method for assay of all foods.[54]

6.3.2.2 *Microbiological*

Microbiological analysis provided some of the earliest methods to assay thiamin from biological matrices. *Lactobacillus fermenti* was originally used, but problems with specificity limit its use. The organism is susceptible to both stimulatory and inhibitory matrix effects. Responses are reported for pentoses, reducing agents, fructose, maltose, calcium, and glucose heat degradation products.[45,56] Successful use of *L. fermenti* has been reported in 96-well microtitre plate assays of biological fluids and foods.[57,58] Deibel et al.[59] introduced *Lactobacillus viridescens* (ATCC 12706) as an improved assay for thiamin. *L. viridescens* is much more specific for thiamin, requiring intact thiamin for growth. The organism is not as subject to matrix effects as *L. fermenti*. Table 6.5 summarizes responses of several bacteria and protozoans used in the past for thiamin assay. Analytical data obtained from the *L. viridescens* assay corresponds closely to properly run thiochrome assays. The organism is widely accepted

Table 6.5 Microbiological Responses Induced by Thiamin and Related Compounds Relative to Thiamin

Organism	Thiamin	Pyrimidine	Thiazole	TPP
Lactobacillus viridescens	1.0	0	0	1.0
Saccharomyces cerevisiae	1.0	0	0	0.7
Ochromonas malhamensis	1.0	0	0	1.0
Lactobacillus fermenti	1.0	0[a]	0[a]	0.3

[a] Response after long incubation periods.

Source: Adapted from Voigt, M.N. and Eitenmiller, R.R., *J. Food Protect*., 41, 730, 1978.

for food analysis.[18] AOAC International does not provide an official microbiological assay for thiamin. The *L. viridescens* assay was recommended for collaboration,[54] but the collaborative study was not completed.

Extraction procedures for the microbiological analysis of thiamin generally follow the thiochrome analysis procedures (AOAC International Method 942.23). Enzyme hydrolysis of the phosphate esters is necessary to avoid differential growth response to TMP, TPP, and TTP.

6.3.2 *Advances in the analysis of thiamin*

6.3.2.1 *Spectroscopic, electrochemical, and capillary electrophoretic methods*

Several recently published procedures using flow-injection analysis (FIA) coupled to spectrophotometric fluorescence or chemiluminescence detection are available (Table 6.6).[60–70] Fluorescence based methods[62–66] rely on conversion of thiamin to highly fluorescent derivatives including thiochrome or closely related fluorescent compounds. The FIA fluorimetric methods are highly sensitive and provide high sample throughput. Chemiluminescence-based methods,[67–70] likewise, show high sensitivity and selectivity. Work by Son and Wu[67,68] relied on the ability of thiamin to linearly decrease chemiluminescence produced from the luminol–ferricyanide reaction or the luminol–KIO_4 reaction. These methods were applicable to pharmaceuticals and biologicals.

Electrochemical[71,72] and capillary electrophoretic[73–79] methods have not been extensively utilized for thiamin assay. While proven to be feasible, the simplicity and proven reliability of spectroscopic and excellent liquid chromatography (LC) methods have deemphasized other approaches. Nevertheless, capillary electrophoresis is a very versatile analytical tool for thiamin. Shabangi and Sutton[79] used capillary zone electrophoresis to assay thiamin and the phosphate esters with a high degree of accuracy.

6.3.2.2 *Liquid chromatography*

For the past several decades, LC has been commonly used for the analysis of thiamin. Reviews on LC methodology include those given in Section 6.3 and a review by Lynch and Young.[80] Table 6.7 provides details on selected papers that provide a historical perspective of method development and recent research approaches.[81–112] Because thiamin and riboflavin can be conveniently assayed concurrently or simultaneously, many methods are available to assay the two vitamins from the same extract. These procedures are discussed in Chapter 14.

6.3.2.2.1 Extraction procedures for the analysis of thiamin by LC. Extraction protocols depend on the investigator's need to quantitate total thiamin or thiamin and the phosphate esters. To assay total thiamin, the extraction must liberate thiamin from the sample matrix and hydrolyze the esters. Such extractions use acid and enzyme hydrolysis that closely follow AOAC International Method 942.23. Recently, two multilaboratory studies were conducted that gave extraction parameters for the concurrent extraction of thiamin and riboflavin. In a French study conducted by the Commission Generale d'Unification des Methods d'Analysis by Arella et al.,[47] a procedure originally reported by Hasselmann et al.[45] was used. Hydrolysis with 0.1 N HCl was followed by enzyme digestion with a combination of β-amylase and takadiastase. The method gave high recoveries except for chocolate powder, which required an increase in HCl concentration to 0.5 N to obtain reliable results.

The European Union Measurement and Testing Program published details of an optimal extraction procedure developed from earlier studies.[114] Steps in the extraction were the following:

1. Autoclave a 0.2–5 g sample in 0.1 N HCl for 30 min at 121°C. Sample size and volume were chosen by laboratories in the study.

Table 6.6 Selected Spectroscopic, Electrochemical, and Capillary Electrophoretic Methods for Thiamin Analysis

Matrix	Method description	Accuracy/precision	References
Spectrophotometric			
Multivitamins	FIA Thiochrome produced by the oxidation of thiamin hydrochloride by $K_3Fe(CN)_6$ was directly measured at 369 nm, replacing fluorescence measurements	DL—1.0 mg L^{-1} %Recovery—97.5–105	*LRA*, 11, 45, 1999[60]
Turbidimetric			
Multivitamins	FIA Thiamin was precipitated by silicotungstic acid and measured at 420 nm	DL—1.0×10^{-5} mol L^{-1}	*Talanta*, 48, 659, 1999[61]
Fluorometric			
Multivitamins, rice	Thiamin oxidation by H_2O_2 is catalyzed by the mimetic enzyme iron(III) tetrasulfonatophthalocyanine. The oxidation product is assayed by fluorescence, Ex λ = 375, Em λ = 440	DL—4.3×10^{-9} mol L^{-1}	*Analyst*, 124, 771, 1999[62]
Pharmaceuticals	FIA Thiamin is derivatized to a fluorescent derivative by OPT in the presence of 2-mercaptoethanol, Ex λ = 340, Em λ = 450	DL—0.1 ng mL^{-1}	*Mikrochem. Acta*, 134, 83, 2000[63]
Pharmaceuticals	FIA Copper(II) ion in combination with boric acid eliminated AA interference in the assay of thiamin by conversion to a fluorescent compound by NaOH and sodium sulfite, Ex λ = 370, Em λ = 440, throughput = 80 h^{-1}	DL—1.84 µg L^{-1} %RSD—0.32	*Anal. Lett.*, 35, 707, 2002[64]
Soft wheat flour	$K_3Fe(CN)_6$ levels for the conversion of thiamin to thiochrome was optimized	—	*Cereal Chem.*, 80, 238, 2003[55]
Pharmaceuticals	FIA Surface solid-phase spectrofluorimetry Thiochrome was concentrated and separated from the reaction medium by SPE on the surface of octadecyl-alkylated polystyrene/divinylbenzene microbeads (18-PS/DP). Fluorescence of the microbeads was measured at Ex λ = 385, Em λ = 433, throughput = 30 h^{-1}	DL—0.03 µg mL^{-1} %RSD—1.0	*Anal. Sci.*, 19, 289, 2003[65]
Pharmaceuticals, serum, urine	FIA Thiamin is converted on-line to a fluorescent product by UV-irradiation. The photoproduct is retained C_{18} silica gel filling the flowcell. Fluorescence is determined at Ex λ = 367, Em λ = 443. Elution from the solid support was by 60% acetonitrile. Throughput = 36–52 h^{-1} depending on injection volume (300 or 600 µL)	DL—28–55 ng L^{-1} %RSD—1.4–2.4	*Anal. Chim., Acta*, 535, 161, 2005[66]

Continued

Table 6.6 (Continued)

Matrix	Method description	Accuracy/precision	References
Chemiluminescence			
Pharmaceuticals, urine	FIA Chemiluminescence produced by the reaction of luminol and ferricyanide was decreased linearly by thiamin	DL—66 pmol mL^{-1} %RSD < 3.0	*Chem. Anal.*, 47, 747, 2002[67]
Pharmaceuticals, urine	FIA Chemiluminescence produced by the reaction between luminol and periodate was decreased by thiamin. Reactants were immobilized on Amerberlyst A-27 anion exchange resin and eluted with water injection	DL—1.0 pmol μL^{-1} %RSD < 3.0	*J. Pharm. Biomed. Anal.*, 28, 683, 2002[68]
Pharmaceuticals	FIA Chemiluminescence produced by the reaction of luminol with H_2O_2 is enhanced by thiamin, throughput = 90 h^{-1}	DL—0.01 µg mL^{-1} %RSD—1.4	*Talanta*, 57, 661, 2002[69]
Pharmaceuticals	FIA Chemiluminescence produced by the oxidation of UV-degradation products of thiamin by permanganate in acid medium is linear from 0.05 to 84 mg L^{-1}. Throughput = 30 h^{-1}	DL—0.05 mg L^{-1} %RSD—1.3–2.5	*Anal. Lett.*, 37, 3205, 2004[70]
Electrochemical			
Pharmaceuticals	Electrochemical luminescence Thiamin was electrochemically oxidized with rhodamin B as a sensitizer: Electrochemical luminescence signal was generated on a platinum electrode	DL—0.08 µg mL^{-1}	*Anal. Chim. Acta*, 394, 165, 1999[71]
Pharmaceuticals, urine	A stripping volammetric procedure based on coupling of adsorptive accumulation and catalytic effects on the oxidation of thiamin to thiochrome. A stripping analyzer coupled with a mercury drop electrode and polarographic cell with Ag/AgCl-saturated KCl reference electrode and platinum wire counter electrode was required	%RSD—3.2 for urine	*J. Pharm. Biomed. Anal.*, 22, 1047, 2000[72]

Capillary electrophoresis			
Meat, milk	MECC 70 cm × 75 µm, 100 mM sodium tetraborate, 50 mM sodium phosphate, 50 mM SDS, 10% isopropyl alcohol, pH 7.6, 15 kV, 50°C, 254 nm	%Recovery—100 for milk	*Eur. Food Res. Technol.*, 209, 355, 1999;[73] *Milchwissenschaft*, 55, 307, 2000[74]
Biological media (plasma, urine, saliva)	CZE (Z-cell) 80.5 cm × 75 µm, 10 mM phosphate, pH 7.2, 30 kV, 200 nm	DL (µg mL^{-1}) water—0.05 saliva—0.05 plasma—0.8 urine—0.10 %RSD (peak area)—0.5–0.8	*J. Chromatogr.* A, 871, 351, 2000[75]
Multivitamins	MECC 57 cm × 75 µm, 40 mM borate-boric acid, pH 8.5, 40 mM SDS or acetyl-trimethyl ammonium chloride, 15 kV, 254 nm	—	*J. Chromatogr. A*, 950, 241, 2002[76]
Multivitamins	MECC 57 cm × 75 µm, acetonitrile–25 mM aqueous borate (20:80), pH 8.4 with 2% methanol, 25 mM *bis*(2-ethylhexyl)sodium sulfoccinate, 24 kV, 200 nm	—	*J. Chromatogr. A*, 953, 257, 2002[77]
Multivitamins	MECC 48.5 cm × 50 µm, 135 mM SDS in borate buffer (0.2 M), pH 8.0, 12 kV, 270 nm	%RSD—0.1–2.5	*J. Chromatogr. A*, 986, 153, 2003[78]
Multivitamins	CZE 50 cm × 75 µm, 65 mM boric acid–8 mM sodium tetraborate decahydrate, pH 8.24, 30 kV, 200 nm	DL—10^{-4}–6×10^{-4} mM QL—6×10^{-4}–1.2×10^{-3} mM	*J. Pharm. Biomed. Anal.*, 38, 66, 2005[79]

Table 6.7 LC Methods for the Analysis of Thiamin in Foods, Feed, Pharmaceuticals, and Biologicals

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods					
Rice flour/ Thiamin	Takadiastase digestion, centrifuge	Nucleosil C_{18}, 5 μm, 15 cm × 4 mm Mobile phase—isocratic 0.01 M NaH_2PO_4 and 0.15 Na perchlorate, pH 2.2 Flow rate–0.6 mL min^{-1}	Thiochrome Post-column Fluorescence Ex λ = 375 Em λ = 435	—	*J. Chromatogr.* 284, 281, 1984[81]
Various foods/ Thiamin, TMP, TPP, TTP	Add amprolium (IS), homogenize w/5% sulfosalicylic acid. Centrifuge, filter water layer. Clean-up: AG2-X8, anion-exchange.	Perkin Elmer C_{18}, 3 μm, 30 cm × 3 mm Mobile phase—gradient 0.1 M Na_3PO_4, pH 5.5–0.1 M Na_3PO_4, pH 2.6 Flow rate—1 mL min^{-1}	Thiochrome Postcolumn Fluorescence Ex λ = 339 Em λ = 432	DL (pmol) TMP—0.46 Thiamin—0.9	*J. Micronutr. Anal.*, 2, 189, 1986[82]
Infant formula, various foods/ Thiamin, non-phosphorylated	Adjust pH to 1.7–2.0 w/6 N HCl. Adjust pH to 4.0–4.2 w/NaOH, filter and inject filtrate	μBondapak C_{18}, 10 μm, 30 cm × 4.6 mm Mobile phase—isocratic EDTA (2g) + sodium hexane sulfonate (3g) + HAC (15 mL) + MeOH (400 mL), dilute to 2 L w/water. Flow rate—2.5 mL min^{-1}	248 nm	$\%RSD_{Intra}$—1.9–3.9 $\%RSD_{Inter}$—1.9–4.9 %Recovery—99–103	*J. AOAC*, 73, 792, 1990[83]
Various foods/ Thiamin	Hydrolysis w/0.1 M HCl (30 mL) and 6 M HCl (0.1 mL), 121°C for 1 min. Adjust pH to 4.0–4.5. Digest w/takadiastase, 48°C, 3 h Clean-up: Amberlite CG-50, C_{18} Sep-Pak	μBondapak C_{18}, 10 μm, 30 cm × 3.9 mm Mobile phase–isocratic MeOH:HAC:sodium hexane sulfonate (ratio–various) Flow rate—1.0 mL min^{-1}	254 nm	DL (on-column)—0.5 ng	*Z. Lebensm Unters Forsch*, 191, 313, 1990[84]
Various foods/ Thiamin	Add 0.25 N H_2SO_4 (10 mL) to 2 g sample, autoclave, 30 min. Adjust pH to 4.6 w/NaOH:HAC mixture. Digest w/takadiastase, 40°C–45°C, 25 min. Digest w/papain, 40°C–45°C, 2 h. Add TCA, heat at 50–60°C, 5 min. Centrifuge	Mercksorb Si60, 10 μm, 25 cm × 4.5 mm Mobile phase—isocratic Phosphate buffer (pH 5.6):EtOH (100:12) Flow rate—1 mL min^{-1}	Thiochrome Post-column Fluorescence Ex λ = 366 Em λ = 464	—	*Food Chem.*, 43, 393, 1992[85]

Various foods/ thiamin	Add 0.1 N HCl (5g sample/65mL), autoclave 30 min. Adjust pH to 4.5. Digest w/takadiastase, add TCA, dilute and filter. Clean–up: Baker C_{18} SPE column or other C_{18} SPE column	Novapak C_{18}, 4 µm, 15 cm × 3.9 mm Mobile phase—isocratic MeOH:50 mM phosphate, pH 7.0 (30:70) Flow rate—1 mL min^{-1}	Thiochrome Post-column Fluorescence Ex λ = 366 Em λ = 436	DL (on-column)—10 ng	*J. Food Compos. Anal.*, 6, 152, 1993[86]
Dried yeast/ thiamin	Add 1 mL 10% HCl and 80 mL water to 1 g sample. Incubate at 80–85°C, 30 min. Add NaOAC buffer, pH 4.5. Digest w/diatase 45–50°C, 3h. Clean–up: extract w/isobutanol, add chloroaniline (IS).	CAPCELL PAK C_{18}, 6 µm, 15 cm × 4.6 mm Mobile phase—isocratic 0.05 M acetate (pH 3.5):MeCN (85:15) w/0.15% sodium I-octanesulfonate	254 nm	—	*Chromato-graphia*, 39, 91, 1994[87]
Various foods/ thiamin	Add HCl (concentration unknown). Digest, 100°C for 30 min. Adjust pH to 4–4.5, digest w/takadiastase 47°C, 3h. Clean-up: weak anion exchange column.	Lichrosphere 100 RP-18, 5 µm, 12.5 cm × 4 mm Mobile phase—isocratic H_3PO_4–KH_2PO_4 (10^{-2} M, pH 3.5): MeOH (85:15) w/5 µM hexane sulfonic acid and 0.1% triethylamine	254 nm	—	*J. Liq. Chrom. Rel. Technol.*, 19, 2155, 1996[88]
Cooked sausages/ thiamin	Add 60 mL 0.1 N HCl to 10 g ground sample, homogenize, autoclave at 120°C, 20 min. Adjust pH to 4.0–4.5 w/2.5 NaAC. Add 5 mL (6%) Clara-diastase, incubate at 50°C, 3 h. Add 2 mL 50% TCA, heat at 90°C, 15 min. Dilute sample to 100 mL w/sater, filter. Pre-column thiamin oxidation. Clean-up: C_{18} Sep-Pak cartridge	Spherisorb C_8, 5 µm, 25 cm × 4 mm Mobile phase—Isocratic phosphate buffer (5 mM, pH 7.0): MeCN (70:30) Flow rate—0.65 mL min^{-1}	Thiochrome Pre-column Fluorescence Ex λ = 360 Em λ = 430	DL—0.15 mg g^{-1} %RSD_{Intra}—1.5 %RSD_{Inter}—5.2 %Recovery >90	*J. Agric. Food Chem.*, 47, 170, 1999[89]
Beers and raw material/ thiamin, TMP, TPP	Liquid sample—1:1 dilution w/25 mM phosphate buufer (pH 7), filter. Solid sample–add 5 mL TCA (20 g L^{-1}) to ground sample (0.02–1 g), sonicate 15 min, centrifuge 15 min. Keep supernatant, reextract twice w/2 mL TCA. Combine the supernatant and dilute to 25 mL w/phosphate buffer. Filter	RP–Amide C_{16}, 5 µm, dimension of the column not reported. Mobile phase—isocratic 25 mM phosphate buffer (pH 7) Flow rate—1 mL min^{-1}	Thiochrome Post-column Fluorescence Ex λ = 375 Em λ = 465	DL—0.06–1.0 ng mL^{-1} QL—0.2–3.4 ng mL^{-1} %RSD—2.0–3.6	*J. Agric. Food Chem.*, 51, 3222, 2003[90]

Continued

Table 6.7 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods					
Dairy products/ thaimin	Add 30 mL 0.1 N HCl to sample (5 mL or 5 g), mix. Autoclave at 125°C for 15 min. Adjust pH to 4.0–4.5 w/2 N NaAC. Add 5 mL Clara-diastase and incubate at 50°C, 3 h. Add 1 mL TCA (50%), incubate at 90°C, 15 min. Pre-column thiamin oxidation Clean-up: C_{18} cartridge	Nucleosil 100-5 C_{18} AB, 12.5 cm × 4 mm Mobile phase—phosphate buffer (0.005 M, pH 7):Methanol (65:36) Flow rate—0.5 mL min^{-1}	Thiochrome Precolumn Fluorescence Ex λ = 360 Em λ = 425	—	*Int. J. Food Sci. Nutr.*, 55, 345, 2004[91]
Feed					
Rodent feed/ thiamin	Add 0.1 N HCl (20 mL) to 1 g sample. Heat at 100°C, 30 min, centrifuge. Adjust aliquot to pH 4.0 w/HAC	SynChropak SCD-100, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:water (40:60) containing 0.05 M pentane sulfonate, adjust pH 4.0 w/HAC Flow rate—0.5 mL min^{-1}	Thiochrome Postcolumn Fluorescence Ex λ = 370 Em λ = 430	DL (on-column)—5 pg	*J. AOAC Int.*, 78, 307, 1995[92]
Pharmaceuticals					
Multivitamins/ thiamin	Dissolve in 0.1 M NaOH, shake 15 min and centrifuge. Dilute w/mobile phase	Polymeric RP Jones Chromatography, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:0.02 M phosphate buffer, pH 11.0 (20:80) Flow rate—2 mL min^{-1}	EC	—	*Analyst*, 120, 1059, 1995[93]
Tablet, capsule, urine/thiamin, TMP, TPP	Capsule/Tablet: dissolve sample w/water and filter. Urine: filter sample	RP-AmideC_{16}, 5 µm, 15 cm × 4 mm Mobile phase—gradient 25 mM KH_2PO_4 (pH 7):MeCN Flow rate—1 mL min^{-1}	230 nm	DL—0.005–0.07 µg mL^{-1} QL—0.02–0.2 µg mL^{-1} %RSD—1.1–2.1 %Recovery—98	*J. Chromatogr. B*, 757, 301, 2001[94]

Biologicals					
Blood, erythrocytes, plasma/ thiamin, TMP, TPP, TTP	Add 0.1 mL TCA (100 g L^{-1}) to 0.2 mL sample, vortex and centrifuge	ISA-07/S2504 Shimadzu, 25 mm × 0.4 mm Mobile phase—isocratic 0.7 M NaOAC Flow rate—0.5 mL min^{-1}, 25°C	Thiochrome Postcolumn Fluorescence Ex λ = 375 Em λ = 435	—	*J. Chromatogr.*, 188, 417, 1980;[95] 245, 141, 1982;[96] Clin. Chem., 29, 2073, 1983[97]
Excitable tissues/ thiamin, TMP, TPP, TTP	Homogenize in 5% TCA, centrifuge. Extract supernatant w/water saturated Et_2O. Water phase reacted form thiochrome	Ultrasphere-ODS, 5 µm, 15 cm or 25 cm × 4.6 mm Mobile phase—gradient 25 mM phosphate buffer (pH 8.4): MeOH	Thiochrome Precolumn Fluorescence Ex λ = 390 Em λ = 475	—	*J. Chromatogr.*, 307, 283, 1984[98]
Standards/ thiamin, TMP, TPP, TTP	–	PRP-1, 10 µm, 25 cm × 4.1 mm Mobile phase—isocratic 25 mM phosphate buffer, pH 8.4	Thiochrome Postcolumn Fluorescence Ex λ = 375-400 filter Em λ = 460-600 filter	—	*J. Chromatogr.*, 295, 486, 1984[99]
Animal tissue/thiamin, TMP, TPP, TTP	Homogenize w/10% TCA; centrifuge. Extract w/water-saturated Et_2O. Lyophilize	CLC-ODS, 15 cm × 6 mm Mobile phase—isocratic 100 µM phosphate buffer (pH 2.5): MeOH (92:8) Flow rate—0.5 mL min^{-1}	Thiochrome Pre-column Fluorescence Parameters not reported.	—	*J. Chromatogr.*, 450, 317, 1988[100]
Blood tissue/ thiamin	Add 0.19 M HCl (blood) or 0.1 M HCl (tissue). Hold at 100°C, 1h. Cool to 37°C. Hydrolyte w/papain and takadiastase. Form thiochrome fluorophores.	Lichrosorb Li60, 5 m, 25 cm × 4 mm Mobile phase—isocratic $CHCl_3$:MeOH (80:20) Flow rate—2 mL min^{-1}	Thiochrome Precolumn Fluorescence Ex λ = 375 Em λ = 430	—	*J. Micronutr. Anal.*, 7, 147, 1990[101]
Blood serum/ thiamin, TMP, TPP, TTP	Add 0.25 mL 2.44 M TCA to 2 mL. Allow to stand 1 h in dark, centrifuge. Extract w/water-saturated Et_2O. Form thiochrome fluorophores	Supelcosil NH_2, 25 cm × 4.6 mm Mobile phase—MeCN:85 mM phosphate buffer (pH 7.5) (90:10) for thiamin and (60:40) for phosphate esters Flow rate—1.5 mL min^{-1}	Thiochrome Precolumn Fluorescence Ex λ = 375 Em λ = 450	—	*J. Chromatogr.*, 564, 127, 1991[102]

Continued

Table 6.7 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Biologicals (*Continued*)					
Neurons, astrocytes/ thiamin, TMP, TPP, TTP	Disrupt cells w/60% TCA. Extract w/Et_2O. Derivatize to thiochrome	PRP -1 5 µm, 15 cm × 4.1 mm Mobile phase—isocratic 50 mM phosphate containing 25 mM TBAHS and 4% THF Flow rate—0.5 mL min^{-1}	Thiochrome Precolumn Fluorescence Ex λ = 365 Em λ = 433	DL—50 fmol for TTP	*Anal. Biochem.*, 198, 52, 1991[103]
Hrythrocytes/ thiamin, TMP, TPP, TTP	Deproteinate w/100% TCA, 0.5 mL TCA + 0.5 mL hemolysate. Centrifuge. Extract w/Et_2O	µBondapak C_{18} 10 µm, 30 cm × 3.9 mm Mobile phase—isocratic 0.15 mM citric acid (pH 4.2):0.4% diethylamine in 0.1 M formic acid (90 :10)	Thiochrome Postcolumn Fluorescence Ex λ = 365 Em λ = 435	%CV—2.6–11.5	*J. Chromatogr.* B, 653, 217, 1994;[104] *Clin. Chim. Acta*, 234, 91, 1995[105]
Plasma/ thiamin, TMP, TPP, TTP as thiamin	Mix 1 mL plasma w/0.15 mL 3 M perchloric acid, vortex and centrifuge. Add 0.3 mL 1 M NaOAC: HAC buffer (pH 4.6) containing 2.4 g NaOH per 100 mL to supernatant. Digest w/acid phosphatase, 16h, 40°C. Add 0.15 mL 3 M perchloric acid and centrifuge	Nucleosil 1205, C_{18}, 12.5 cm × 4 mm Mobile phase—Isocratic MeCN:(10 mM perchloric acid - 10 mM octane sulfonic acid) (25:75) Flow rate—2 mL min^{-1}	Thiochrome Postcolumn Fluorescence Ex λ = 365 Em λ = 435	Precision %CV—0.9–4.3 Reproducibility %CV—1.8–4.3	*Meth. Enzymol.*, 279, 83, 1997[106]
Human erythrocytes/ thiamin, TMP	Add 250 µL 40% TCA to 2 mL cell suspension, mix and stand at dark, 1h. Centrifuge, 20 min. Transfer liquid phase. Extract w/Et_2O. Derivatize to thiochrome	Ultra Amino 5 µm, 100 Á, 25 cm × 4.6 mm Mobile phase—isocratic Potassium phosphate buffer (pH 7.5, 85 mM)–acetonitrile (65:35) Flow rate—1 mL min^{-1}	Thiochrome Precolumn Fluorescence Ex λ = 375 Em λ = 430	DL—1 nmol L^{-1} QL—2.5 nmol L^{-1} %RSD_{Intra}—5 %RSD_{Inter}—9 %Recovery—102	*J. Chromatogr.* B, 789, 355, 2003[107]

Serum, urine/ thiamin	No sample preparation—Inject 300 µL undiluted urine or serum into HPLC system (polyethylene column)	Polyethylene column, 50 × 4.6 mm Mobile phase—gradient Water-sodium phosphate—methanol	Thiochrome Postcolumn Fluorescence Ex λ = 365 Em λ = 435	DL—1.6 µg L^{-1} QL—9.6 µg L^{-1} %Recovery—84.8–98.8 %RSD—3.6–14.5	*Microchem. J.*, 78, 71, 2004[108]
Serum, urine, erythrocytes/ thiamin, TMP, TTP	Plasma, erythrocytes—Add 7.2% perchloric acid to the sample (1:1, v/v), mix, ice bath, 10 min; again, mix, ice bath for 5 min. Centrifuge, keep supernatant Urine—dilute sample 1:5 w/water. Derivatize to thiochrome	Microshpere C_{18}, 3 µm, 10 cm × 4.6 mm Mobile phase—gradient A–KH_2PO_4 (0.2 M)—TBAH (0.3 mM, pH 7):MeOH (88.5:11.5) B–MeOH:H_2O (70:30) Flow rate—1 mL min^{-1}	Thiochrome Precolumn Fluorescence Ex λ = 365 Em λ = 435	DL—0.2–0.4 ng mL^{-1} QL—0.6–1.2 ng mL^{-1} %Recovery—85–112	*J. Pharm. Biomed. Anal.*, 37, 1025, 2005[109]
Rat tissues/ thiamin, TMP	Add 7 mL perchloric acid to 1g sample, homogenize and centrifuge. Derivatize the supernatant immediately	RP-amide C_{16}, 5 µm, 15 cm × 4 mm Mobile phase—isocratic Potassium phosphate buffer (50 mM, pH 6):MeOH (80:20). Flow rate—1 mL min^{-1}	Thiochrome Precolumn Fluorescence Ex λ = 366 Em λ = 435	$\%RSD_{Intra}$—≤ 9 $\%RSD_{Inter}$—≤ 9.4 %Recovery—80–96	*J. Chromatogr.* B, 816, 67, 2005[110]
Seawater/ thiamin	Preconcentrate by SPE (C_{18} resin). Elute w/MeOH. Evaporate aliquote, redissolve in MeOH	Premier C_{18}, 5 µm, 15 cm × 4.6 mm Mobile phase—gradient Solvent A—0.05 M NH_4OAC Solvent B—MeOH A:B (92.5:7.5) to (70:30) over 15 min Flow rate—1 mL min^{-1}	270 nm	%Recovery—93–98	*Limol. Oceanogr.: Methods*, 3, 241, 2005[111]
Seawater, microalgal media/ thiamin	Preconcentrate by SPE (Sep-Pak C_{18}). Elute w/MeOH. Evaporate. Redissolve in 0.1 mol L^{-1} HCl. Centrifuge	Sperisorb ODS 2, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:0.05 NaOAC, pH 8.5 (40:60) Flow rate—1 mL min^{-1}	Thiochrome Precolumn Fluorescence Ex λ = 375 Em λ = 440	DL—1×10^{-10} mol L^{-1} %RSD—3.1	*Anal. Bioanal. Chem.*, 383, 875, 2005[112]

2. Adjust an aliquot to pH 4.0 with 4.0 M sodium acetate buffer (pH 6.1).
3. Add 100 mg takadiastase per g sample.
4. Incubate at 37–45°C for 4 h for thiamin (18 h for riboflavin).
5. Cool, filter, or centrifuge.

At this point, the extract was ready for either microbiological or LC analysis. This extraction procedure uses a high ratio of enzyme to sample, which could produce high enzyme blanks.

Various methods developed for specific matrices have advantageously used solid-phase extraction (SPE) to concentrate and/or cleanup extracts before assay.[84,86,88,89,91,111,112] SPE on C_{18} resin or commercial column C_{18} SPE columns are the common choice. Okbamichael and Sañudo-Wilhelmy[111] and He et al.[112] applied SPE on C_{18} to concentrate thiamin from seawater between assays by passing quite large volumes through the SPE column before elution. He et al.[112] passed 1 L of seawater through each column with excellent recoveries (89–110%).

Ndaw et al.[114] developed an enzyme extraction using α-amylase, papain, and acid phosphatase for the combined extraction of thiamin, riboflavin, and vitamin B_6 from foods before LC assay. The digestion eliminated the need for acid hydrolysis to free bound forms of the vitamins.

Quantitation of free thiamin and the individual phosphate esters requires conditions that do not hydrolyze the ester bonds. Sample extraction is limited to acid hydrolysis to free matrix-bound thiamin.[82] Enzyme digestion common to thiochrome and microbiological assay is eliminated. Sulfosalicylic acid has been successfully used for extraction of free thiamin, TMP, TPP, and TTP from foods.[82] Further extract cleanup by SPE, solvent partitioning, and protein precipitation is common. For blood, serum, urine, and other biological fluids, acid hydrolysis is not necessary. For these matrices, the sample is deproteinized with TCA or perchloric acid. If TCA is used, the extract is treated with water-saturated diethyl ether to remove excess TCA. Tissue samples are often digested with protease to aid liberation of thiamin.

6.3.2.2.2 Chromatography parameters

6.3.2.2.2.1 Supports and mobile phases. Reversed-phase chromatography on C_{18} support with an isocratic mobile phase works efficiently for the resolution of thiamin, TMP, TPP, and TTP. While C_{18} has been the most common support for thiamin chromatography, polystyrene resin provides advantages when precolumn thiochrome procedures are used. Various investigators have used PRP-1 (polystyrene-divinyl-benzene beads) for chromatography of the thiochromes. Bontemps et al.[98] observed that silica-based C_{18} supports quickly degraded at pH 8.4 and that poly(styrene-divinyl-benzene) resins could be used for reversed-phase resolution of thiochrome products of thiamin and the phosphate esters at higher mobile phase pH levels. These authors subsequently reported that 10 mm PRP-1 was useful for the chromatography at pH 8.4, which was sufficiently close to the fluorescence optima of the thiochromes to provide excellent sensitivity.[99] More polar NH2 supports have been quite effectively used for resolution of precolumn formed thiochromes of the phosphorylated vitamers.

Application of silica-based C_{18} supports for precolumn thiochrome procedures was facilitated with the use of an acid mobile phase of 100 mM NaH_2PO_4–H_3PO_4 buffer, pH 2.5—8% methanol.[100] To provide for maximal fluorescence of the thiochrome products, 0.2 M NaOH—70% methanol was pumped into the column effluent to raise the pH to 8.6. The procedure developed by Iwata et al.[100] is given in detail in Section 6.4.

Mobile-phase components of published reversed-phase systems suitable for thiamin resolution include methanol–water, and methanol buffers (phosphate and acetate),

acetonitrile–buffers, and buffers of various concentrations. Ion-pair reagents, such as heptane or hexane sulfonates and tetrabutylammonium hydroxide, are often added to the mobile phase to improve resolution. Gradients, while not necessary, have been successfully used for the chromatography. Mobile phases for the methods outlined in Table 6.7 indicate the variability in chromatography systems that have been efficiently used for LC resolution of thiamin and its esters from complex sample digests.

6.3.2.2.2.2 Detection. Ultraviolet (UV) detection at 245–254 nm can be used for high potency samples such as pharmaceuticals or enriched foods. However, lack of sensitivity and selectivity precludes its use for most LC separations. UV detection is not sensitive enough for naturally occurring levels of thiamin, TMP, TPP, and TTP in foods and most biological samples. Further, extensive extract cleanup and concentration is required to allow detection by UV.

The utility of thiochrome as a specific and highly fluorescent product of thiamin oxidation has led to the routine use of precolumn and postcolumn thiochrome procedures for fluorescence quantitation of thiamin and its esters. Thiochrome-formed precolumn refers to procedures that form the thiochromes directly in the sample extract. The thiochrome products are chromatographically resolved. Precolumn thiochrome procedures are simpler to set up compared to postcolumn systems. To maximize fluorescence (Ex λ 365–375, Em λ 425–435), pH of the mobile phase must be kept above 8.0.[30] C_{18} reversed-phase columns are quickly degraded under the high pH environment. As discussed in Section 6.3.3.2.1, polystyrene-divinylbenzene supports help overcome this obstacle. Batifloulier et al.[110] assayed thiamin and thiamin phosphate esters by precolumn thiochrome formation and subsequent chromatography on an RP-amide C_{16} column with complete resolution of TPP and TTP. The method is provided in Section 6.4.

Postcolumn procedures are based on the conversion of thiamin compounds to their respective thiochromes after chromatographic resolution of the thiamin forms. Postcolumn oxidation requires an additional pumping system to deliver the oxidizing agent, which is usually 0.01% potassium ferricyanide in 15% sodium hydroxide. The oxidizing agent is pumped into the column effluent and sent through a mixing coil ahead of the fluorescence detector. Bohrer et al[108] used postcolumn conversion of thiamin to thiochrome to analyze serum and urine. The samples were directly injected into the LC system without cleanup or dilution. Thiamin was separated from the sample matrix components on an ultrahigh molecular weight surface-modified polyethylene support with a water–phosphate buffer gradient before introduction of hexacyanoferrate solution for thiochrome development. Postcolumn methods provide resolution and sensitivity in the fentomole range equivalent to precolumn methods.

6.3.2.2.2.3 Internal standards. The general consensus of researchers involved in the development of LC methods for thiamin analysis is that adequate internal standards are not available. Salicylamide, sodium salicylate, and anthracene fluoresce similarly to thiochrome and have been used for injection volume correction.[30] Amprolium was reported to be a usable internal standard to carry through the thiochrome reaction[82]; however, Ollilainin et al.[86] found that amprolium produced an unknown compound during oxidation to form thiochrome products with a K′ value over 60. Band broadening of the amprolium thiochrome product also occurred. These investigators concluded that amprolium could not be used reliably as an internal standard for thiochrome. Recently, phenacetin was applied to the UV detection of thiamin; however, the compound was added immediately before injection and was not a true internal standard.[87] Owing to the nonavailability of suitable internal standards, external standard methods are routinely used for quantification.

6.4 *Method protocols*

Improved High-Performance Liquid Chromatographic Determination of Thiamin and Its Phosphate Esters in Animal Tissues

J. Chromatogr., 450, 317, 1988[100]

Principle

Thiamin, TMP, TDP, and TTP were converted into thiochrome fluorophores by precolumn alkaline oxidation with cyanogen bromide. Thiochromes were resolved by reversed-phase chromatography on octyldecylsilica (ODS) using an acidic mobile phase. Alkaline methanol was mixed with the column effluent before the fluorescent detector.

Chemicals

- Trichloroacetic acid (TCA)
- Diethyl ether
- Cyanogen bromide
- Sodium hydroxide
- Hydrochloric acid
- Thiamin monophosphate
- Thiamin diphosphate
- Thiamin triphosphate

Apparatus

- Liquid chromatograph
- Fluorescent detector
- Proportioning pump

Procedure

Animal Tissue Extraction

- Homogenize with cold TCA.
- Centrifuge (16,000 × g), 15 min.
- Extract TCA with water-saturated diethyl ether.
- Lyophilize, store at 220°C.

Thiochrome Formation

- Divide extract into two fractions, A and B.
- A:
  - To 200 mL A, add 25 mL 0.3 M CNBr
  - Vortex 1 min
  - Add 25 mL 1 M NaOH
  - Vortex 1 min
  - Neutralize with 3 M HCl.
- B:
  - To 200 mL B, add 25 mL 1 M NaOH.
  - Vortex 1 min.
  - Add 25 mL 0.3 M CNBr.
  - Vortex 1 min.
  - Neutralize with 3 M NaOH.

Note: B serves as the blank. Addition of the NaOH destroys thiamin compounds. Chromatography of the blank will indicate possible nonthiochrome fluorescent compounds that are not resolved from the thiamin derivatives.

Chromatography

Mobile phase	100 mM sodium dihydrogen phosphate–phosphoric acid buffer, pH 2.5, 8% methanol
Column	15 cm × 6 mm
Stationary phase	Shimpak CLC-ODS
Column temperature	50°C
Flow rate	0.5 mL min^{-1}
Injection volume	20–40 mL
Alkali delivery	0.2 M NaOH:Methanol (30:70) at 0.5 mL min^{-1}, CRB-IB incubator box at 50°C
Detection	Fluorescence, excitation, and emission at wavelengths suitable for thiochrome
Calculation	External standard, peak area, linear regression

Determination of Thiamin and Its Phosphate Esters in Rat Tissues Analyzed as Thiochromes on a RP-Amide C16 Column
J. Chromatogr. B, 816, 67, 2005[110]

Principle

Thiamin and its phosphate esters were converted to thiochrome. Thiochromes were resolved on RP-amide C16.

Chemicals

- Thiamin
- Thiamin monophosphate
- Thiamin diphosphate
- $K_3Fe(CN)_6$
- NaCl
- KH_2PO_4
- HCl
- $HClO_4$
- K_2CO_3
- Milli-Q water

Apparatus

- Polytron homogenizer
- Liquid chromatograph
- Fluorescence detector
- Centrifuge

Tissue Extraction

- Homogenize 1 g in 7 mL 0.4 M $HClO_4$
- Centrifuge, 8000 g, 10 min

Thiochrome Formation

- Add 20 µL of 30.4 M $K_3Fe(CN)_6$ in 15% NaOH to 200 µL of the perchloric extract
- Mix by Vortex for 10 s and allow to stand 60 s
- Add 5 µL of 15% NaOH

Chromatography

Mobile phase	50 mM phosphate buffer, pH 6.0—Methanol (80:20)
Column	15 cm × 4 mm (Precolumn, 2 cm × 4 mm)
Stationary phase	RP-amide C16, 5 µm
Flow rate	1 mL min^{-1}
Injection volume	20 µL
Detection	Fluorescence, Ex λ = 366, Em λ = 435
Calculation	External standards, peak area

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chapter seven

Riboflavin

7.1 Review

Riboflavin (Vitamin B_2) was isolated in 1932 from Brewer's yeast as a yellow enzyme thought to function in cell respiration. From 1933 to 1935, the structure was determined and riboflavin was synthesized. The coenzymes, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) were characterized from 1934 to 1938. Riboflavin deficiency or ariboflavinosis usually occurs along with other water-soluble vitamin deficiencies. The close relationship of riboflavin metabolism with the metabolism of vitamin B-6, folic acid, and niacin leads to deficiency symptoms that are often interrelated or the result of improper functioning of metabolic systems requiring other nutrients.[1,2] Generalized symptoms result from the involvement of FMN and FAD in many aspects of energy, protein, and lipid metabolism. A characteristic symptom of riboflavin deficiency is oral-buccal cavity lesions including angular stomatitis (characteristic fissures at the corners of the mouth). Other symptoms include seborrheic dermatitis, scrotal and vulvar skin changes, cheilosis, and monocystic anemia.[1]

Biochemical status tests are usually required to substantiate riboflavin deficiency. Subclinical deficiency is assessed by measurement of erythrocyte glutathione reductase (EGR), which requires FAD as the cofactor. The enzyme catalyzes the conversion of glutathione to reduced glutathione with the participation of NADPH. The assay follows the *in vitro* stimulation of EGR activity through the addition of FAD. The spectrophotometric assay results are converted to an activity coefficient, which rises in subjects to a riboflavin intake level of 0.5 mg 1000 $kcal^{-1}$.[3]

$$\text{EGR AC} = \frac{\text{Activity (with added FAD)}}{\text{Basal Activity (without added FAD)}}$$

Riboflavin levels in urine based on 24 h urine collections can be used to confirm EGR observations. As for thiamin, riboflavin concentrations in blood, serum, and erythrocytes are not dependable measures of status owing to subject variability.

Good dietary sources of riboflavin include most animal products. Milk and other dairy foods are excellent natural sources. Cereals, unless fortified, are low but consistent sources. Green vegetables are generally higher in riboflavin compared with fruits, which contain lower amounts of the vitamin. Data provided in the USDA National Nutrient Database for Standard Reference, Release 19[4] show that fortified breakfast cereals and enriched cereals are concentrated riboflavin sources. Milk and dairy products, because of the excellent natural riboflavin levels, are also significant food sources (Table 7.1). Cooking can result in large losses through leaching of riboflavin into the cooking medium. In addition, light exposure can lead to significant losses during storage. Dietary Reference Intake (DRI) values

Table 7.1 Riboflavin Content of Various Foods

Description	NDB No	mg 100 g^{-1}	Description	NDB No	mg 100 g^{-1}
Cereals ready-to-eat, GENERAL MILLS, Whole Grain TOTAL	08077	8.06	Wheat flour, white, bread, enriched	20083	0.51
Cereals ready-to-eat, KELLOGG, KELLOGG'S Complete Wheat Bran Flakes	08028	5.90	Wheat flour, white, all-purpose, enriched, bleached	20081	0.49
Cereals ready-to-eat, KELLOGG, KELLOGG'S PRODUCT 19	08058	5.70	Wheat flour, white, cake, enriched	20084	0.43
Cereals ready-to-eat, GENERAL MILLS, TOTAL Corn Flakes	08246	5.67	Milk, canned, condensed, sweetened	01095	0.42
Cereals ready-to-eat, GENERAL MILLS, TOTAL Raisin Bran	08247	3.09	Wheat flour, white, all-purpose, self-rising, enriched	20082	0.41
Cereals ready-to-eat, GENERAL MILLS, FROSTED WHEATIES	08266	2.83	Cornmeal, degermed, enriched, yellow	20022	0.41
Cereals ready-to-eat, GENERAL MILLS, WHEATIES	08089	2.83	Cornmeal, self-rising, degermed, enriched, yellow	20025	0.39
Cereals ready-to-eat, KELLOGG, KELLOGG'S ALL-BRAN Original	08001	2.71	Pie crust, standard-type, frozen, ready-to-bake, baked	18335	0.38
Cereals ready-to-eat, GENERAL MILLS, CHEERIOS	08013	1.68	Milk, canned, evaporated, nonfat	01097	0.31

Braunschweiger (a liver sausage), pork	07014	1.53	Soybeans, mature, cooked, boiled, without salt	16109	0.28
Cereals ready-to-eat, KELLOGG, KELLOGG'S FROSTED FLAKES	08069	1.50	Breakfast items, biscuit with egg and sausage	21005	0.25
Turkey, all classes, giblets, cooked, simmered, some giblet fat	05172	1.50	Yogurt, plain, skim milk, 13 g protein per 8 ounce	01118	0.23
Cereals ready-to-eat, QUAKER, QUAKER OAT LIFE, plain	08049	1.46	Yogurt, plain, low fat, 12 g protein per 8 ounce	01117	0.21
Cereals ready-to-eat, GENERAL MILLS, TRIX	08078	1.42	Cheese, ricotta, whole milk	01036	0.20
Cereals ready-to-eat, GENERAL MILLS, COCOA PUFFS	08271	1.42	Milk shakes, thick vanilla	01111	0.19
Cereals ready-to-eat, GENERAL MILLS, LUCKY CHARMS	08050	1.42	Milk, nonfat, fluid, with added vitamin A (fat free or skim)	01085	0.18
Spaghetti with meat sauce, frozen entrée	22401	1.33	Milk, whole, 3.25% milkfat	01077	0.18
Chicken, broilers or fryers, giblets, cooked, simmered	05022	1.05	Milk, lowfat, fluid, 1% milkfat, with added vitamin A	01082	0.18
Cereals ready-to-eat, KELLOGG, KELLOGG'S RAISIN BRAN	08060	0.72	Milk, reduced fat, fluid 2% milkfat, with added vitamin A	01079	0.18
Fast foods, shrimp, breaded and fried	21059	0.55	Cheese, ricotta, part skim milk	01037	0.18

Source: Data from United States Department of Agriculture, Agricultural Research service, 2006, USDA Nutrient Database for standard Reference, Release 19, Nutrient Data Laboratory Home Page, http://www.nal.usda.gov/fnic/foodcomp, Riverdale, MD, Nutrient Data Laboratory, USDA.

Table 7.2 Dietary Reference Intakes for Riboflavin

Life stage	DRI (mg d^{-1})
Infants (months)	
0–6	0.3
7–12	0.4
Children (years)	
1–3	**0.5**
4–8	**0.6**
Males (years)	
9–13	**0.9**
14–18	**1.3**
19–30	**1.3**
31–50	**1.3**
51–70	**1.3**
>70	**1.3**
Females (years)	
9–13	**0.9**
14–18	**1.0**
19–30	**1.1**
31–50	**1.1**
51–70	**1.1**
>70	**1.1**
Pregnancy (years)	
<18	**1.4**
19–30	**1.4**
31–50	**1.4**
Lactation (years)	
<18	**1.6**
19–30	**1.6**
31–50	**1.6**

Bold type: Recommended dietary allowance; Ordinary type: Adequate intake (AI).

Source: Food and Nutrition Board. Institute of Medicine, *Dietary Reference Intake for Thiamin, Riboflavin, Niacin, Vitamin B_6, Folate, Vitamin B_{12}, Pantothenic Acid, Biotin, and Choline*, National Academy of Sciences Press, Washington, DC, 2000, Chap. 5.

(Table 7.2) were published by the Institute of Medicine in 2000.[2] Recommended Dietary Allowances (RDAs) are based upon clinical observations that an intake of 0.6 mg 1000 $kcal^{-1}$ will prevent deficiency.[1,2] Recommended Dietary Allowances for riboflavin range from 0.5 mg d^{-1} for children (1–3 years) to 1.6 mg d^{-1} for lactating females.[2] The Reference Daily Intake (RDI) used for the nutritional label declaration is 1.7 mg.[5] Aspects of the varied roles riboflavin plays in human health has recently been reviewed by Powers.[6]

The principle form of riboflavin in biological materials is FAD.[7,8] FAD and FMN occur as protein bound flavoproteins in tissue and are primarily bound to albumin in serum. Total riboflavin includes riboflavin, FAD, and FMN.[8] The flavoprotein enzymes function in one- or two-electron oxidation–reduction reactions. Flavin enzymes that act aerobically are oxidases and those that function anaerobically are dehydrogenases. Notable reactions involving the flavins include electron transfer between NADH and the cytochromes, amino acid oxidase reactions, glutathione reductase, succinate and fumarate dehydrogenase, conversion of tryptophan to niacin, pyridine-linked dehydrogenases, acyl-CoA dehydrogenases, β-oxidation of fatty acids, and many other reactions involving oxidation–reduction. FAD participates in the respiratory chain; therefore, it is an integral part of energy production.[9]

7.2 Properties

7.2.1 Chemistry

7.2.1.1 General properties

Riboflavin is 7,8-dimethyl-10-(1′-D-ribityl)isoalloxazine (Figure 7.1). The isoalloxazine ring is commonly known as the flavin ring. The flavin ring is methylated at the 7 and 8 positions. The vitamin is substituted on the 10 position with a D-ribityl side chain. Phosphorylation at the 5′ position of the ribityl side chain produces FMN. Addition of adenosine-5′-monophosphate yields FAD. Structural relationships of riboflavin, FMN, and FAD are shown in Figure 7.2. The carbon–nitrogen bond linking the ribityl side chain to the isoalloxazine is stable to acid hydrolysis; whereas, FMN and FAD are easily converted to riboflavin below pH 5.0.[7]

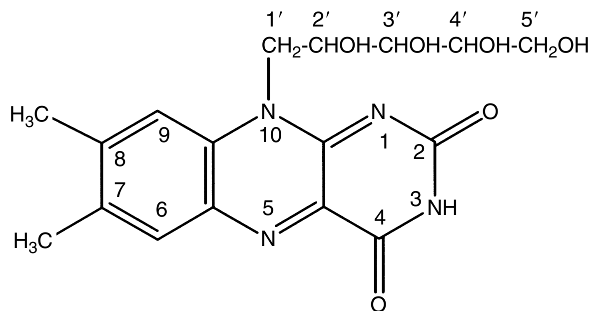

Figure 7.1 Structure of riboflavin.

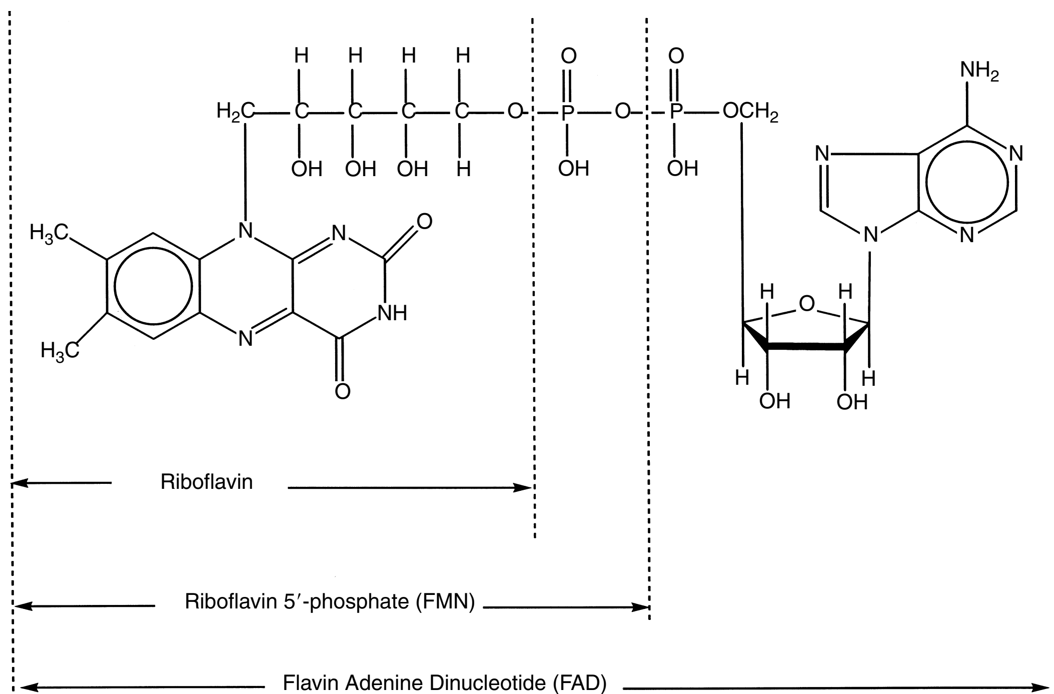

Figure 7.2 Structures of riboflavin and the flavin coenzymes.

For this reason, analytical procedures often incorporate acid hydrolysis as the first step with subsequent quantitation of total riboflavin. Conversely, acid conditions must be avoided in the quantitation of the coenzyme forms.

Physical properties of riboflavin ($C_{17}H_{20}N_4O_6$), FMN ($C_{17}H_{21}N_4O_9P$), which is known commercially as riboflavin monophosphate or riboflavin 5′-phosphate, and FAD ($C_{27}H_{33}N_9O_{15}P_2$) are given in Table 7.3. Riboflavin is available as the USP standard. The vitamin is a yellow-green, fluorescent compound that produces yellow-orange needle-shaped crystals.[13] Riboflavin, FMN, and FAD are slightly soluble in alcohol and insoluble in ether, acetone, benzene, and chloroform. Water-solubility of riboflavin and riboflavin 5′-phosphate differ with riboflavin having only slight solubility in water (0.10–0.13 g L^{-1}) compared to high water-solubility of riboflavin 5′-phosphate (30 g L^{-1}).[9] Water-solubility limits the use of riboflavin to products that do not require rehydration. Riboflavin in high concentrations is bitter. Fat-enrobed forms are available commercially for use in products where off-flavors due to riboflavin addition are a problem.

7.2.1.2 Spectral properties

The characteristic spectral properties of riboflavin, FMN, and FAD provide the basis of chemical assays and the sensitivity and specificity for liquid chromatography (LC) analysis.[14] In aqueous solutions, riboflavin shows absorption maxima of 223, 266, 373, and 445 nm.[15] In the oxidized state, flavins and flavoproteins have absorption maxima at 370 and 450 nm.[6] The maxima shown around 370 nm for riboflavin and the coenzymes is affected by the solvent environment and shifts to lower wavelengths as the polarity of the solvent decreases.[13] Absorption maxima are quite similar for riboflavin and FMN with slight variations being apparent in the spectrum of FAD.[15] The absorption spectrum of riboflavin at pH 7.0 in phosphate buffer is shown in Figure 7.3.[16] Reduction of the flavins and flavoproteins produces atypical and variable absorption spectra above 300 nm that make interpretation of spectroscopic data difficult.[9] Strong absorbance with the characteristic maxima is absent in the reduced states. E1% 1 cm values for riboflavin determined in 0.1 M phosphate buffer, pH 7.0, at 260, 375, and 450 nm are 736, 282, and 324, respectively (Table 7.3).

The ultraviolet (UV)-visible spectral properties have been used for riboflavin analysis; however, most chemical and LC methods capitalize on the strong native fluorescence of the flavins (Ex $\lambda = 440$–500, Em $\lambda = 520$–530).[14] Riboflavin and FMN fluoresce similarly and FAD less strongly. FAD has 10%–20% of the fluorescent intensity on an equimolar basis compared to riboflavin and FMN.[7,11] Interaction of the adenine with the isoalloxazine ring leads to quenching of the fluorescence producing a quantum yield that is approximately ten times less than the quantum yield of riboflavin or FMN.[11] Fluorescence intensity is not affected at intermediate pH levels. Reduced forms of the flavins do not fluoresce.[15] Studies by Sikorska et al.[17] provide data on complex time-dependent density function theory (TD-DFT) of flavin-related compounds and their electronic structure.

7.2.2 Stability

Riboflavin is stable to heat and oxidation if protected from light; thus, most food processing operations have little effect on riboflavin content. Physical loss can be quite extensive through leaching effects, but little chemical degradation is likely to occur. Stability increases as acidity increases. Maximal stability to heat degradation is between pH 2.0 and 5.0. Destruction of the isoalloxazine ring occurs above pH 7.0,[15] and FMN and FAD are converted to riboflavin below pH 5.0.[7]

Riboflavin, FMN, and FAD are easily degraded by UV and visible light with the range of 420–560 nm causing the greatest effect.[18] Photochemical degradation proceeds through photoreduction of the isoalloxazine ring by electrons donated by the ribityl side chain.[7,19]

Table 7.3 Physical Properties of Riboflavin, FMN, and FAD

					Spectral properties				
								Fluorescence	
Substance[a]	Molar mass	Formula	Solubility	Crystal form	λ_{max} nm[b]	$E^{1\%}_{1\,cm}$	$\epsilon \times 10^{-3}$	Ex: nm	Em: nm
Riboflavin Vitamin B_2 CAS No. 83–88-5	376.37	$C_{17}H_{20}N_4O_6$	Soluble but unstable in dilute alkali	Fine yellow-orange needles	260	[736]	27.7	360, 465	521
8367			Slightly soluble in water, 0.10–1.13 g L^{-1}	M.P. 278°C–282°C (dec.)	375	[282]	10.6	pH 3.5–7.5	
			Slightly soluble in alcohol, phenol		450	[324]	12.2		
			Insoluble in $CHCl_3$, acetone, benzene, ether						
Riboflavin-5′-phosphate CAS No. 130–40-5	456.35	$C_{17}H_{21}N_4O_9P$	Soluble in water, 30 g L^{-1} (Na salt)	Fine, yellow-orange crystalline powder	260	[594]	27.1	440–500	530
8368			Insoluble in acetone, benzene, ether	M.P. 280°C–290°C (dec.)	375	[228]	10.4	pH 3.5–7.5	
					450	[267]	12.2		
Flavin-adenine dinucleotide FAD CAS No. 146–14-5	785.56	$C_{27}H_{33}N_9O_{15}P_2$	Soluble in water		260	[471]	37.0	440–500	530
4131			Insoluble in $CHCl_3$, acetone, benzene, ether		375	[118]	9.3	pH 2.7–3.1	
					450	[144]	11.3		

[a] Common or generic name; CAS No.: Chemical Abstract Service number, bold print designates the Merck Index monograph number.

[b] Values in brackets are calculated from corresponding $E^{1\%}_{1\,cm}$ values.

Sources: From Budavari, S., *The Merck Index*, 13th Ed., Merck and Company, Whitehouse Station, NJ, 2001, p. 1410; Nielsen, P., Flavins, In *Modern Chromatographic Analysis of Vitamins*, 3rd ed., De Leenheer, A. P., Lambert, W. E., and Van Boexlaer, J. F., eds., Marcel Dekker, New York, 2000,Chap. 9; Committee on Food Chemicals Codex, *Food Chemicals Codex*, 5th ed., National Academy of Science, Washington, DC, 2004, p. 339; Shah, J. J., Riboflavin, In *Methods of Vitamin Assay*, Augustin, J., Klein, B. P., Becker, D.A., and Venugopal, P. B., eds., John Wiley and Sons, New York, 1985, Chap. 14.

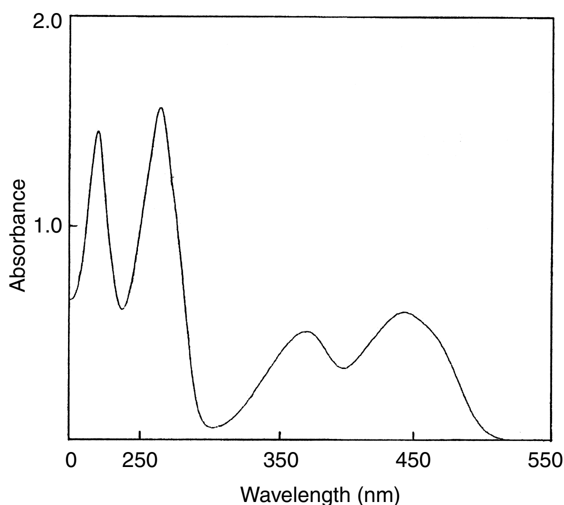

Figure 7.3 Absorption spectrum of 5 × 10^{-5} M riboflavin in pH 7.0 phosphate buffer. (Reproduced from Ahmad, I., Fasihullah, Q., and Vaid, F. H. M., *J. Photochem. Photobiol.*, 82, 21, 2006. With permission.)

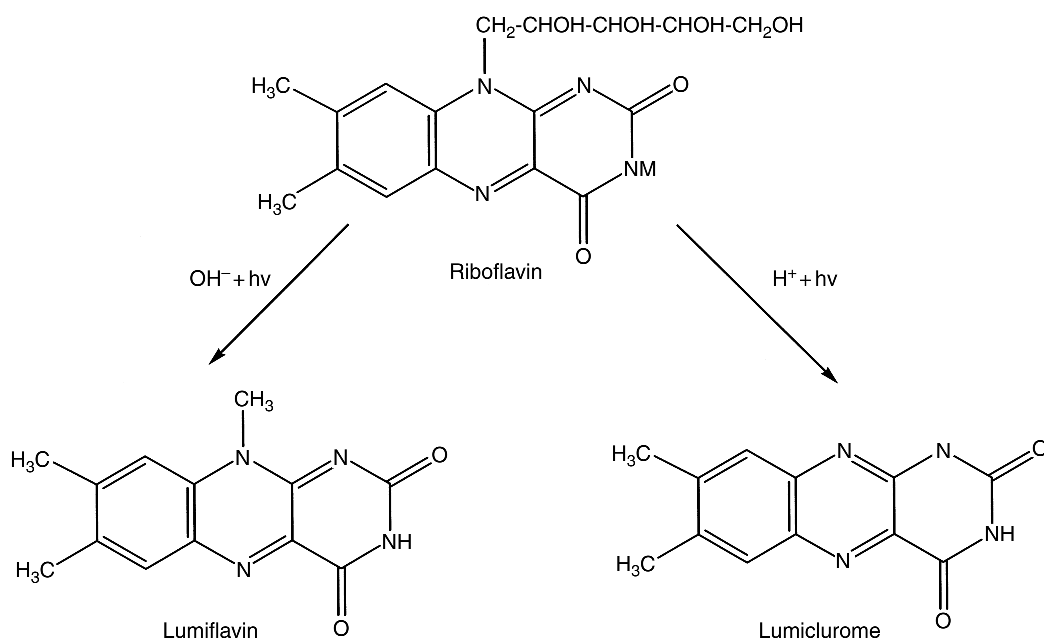

Figure 7.4 Conversion of riboflavin to lumiflavin and lumichrome.

The pH of the solution controls the route of degradation. In acidic or neutral solutions, the ribityl side chain is cleaved, forming lumichrome (Figure 7.4). Under alkaline pH, UV light exposure forms lumiflavin through cleavage at the deoxy-end carbon of the ribityl side chain.[20] Photoaddition primarily occurs under UV radiation and yields cyclodehydroriboflavin (CDRF).[16] The lumiflavin reaction is an important analytical tool to enhance flavin detection in chromatographic analysis, since it fluoresces more strongly than the native riboflavin.[21,22]

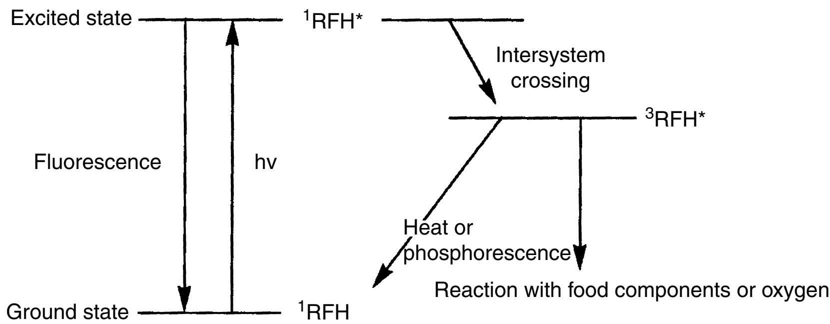

Figure 7.5 Excitation and deactivation of riboflavin under light. ^{1}RFH—singlet state riboflavin, ^{1}RFH*—excited singlet state riboflavin, ^{3}RFH*—excited triplet state riboflavin. (Reproduced from Choe, E., Huang, R., and Min, D., *J. Food Sci.*, 70, R28, 2005. With permission.)

The photochemistry of riboflavin is quite complex because of the ability to accept of lose a pair of hydrogen atoms.[23–30] The ability of riboflavin to generate reactive oxygen species including superoxide anions and singlet oxygen is well understood.[30–33] The roles of riboflavin as a photosensitizer through Type I or Type II mechanisms and as a pro-oxidant in foods has been reviewed by Choe et al.[23] After being light photoenergized, riboflavin is converted into excited triplet state riboflavin. Following Type I reactions, the excited triplet state is reduced by abstraction of electrons or hydrogen ions from other food components to form a riboflavin radical. Type II reactions produce superoxide anion or singlet oxygen through the reaction of the excited triplet state riboflavin with triplet oxygen. Figure 7.5 depicts excitation and deactivation reactions of riboflavin under photosensitization conditions. Jung et al.[34] recently identified 2,3-butanedione as a product of the reaction between riboflavin and singlet oxygen. The product had a buttery odor and was the first reported oxidation product of the reaction between riboflavin and singlet or triplet oxygen in food. The buttery odor potentially could affect flavor quality of high riboflavin-containing foods.

The high sensitivity of flavins to light degradation is a significant factor influencing food packaging. The light induced loss of riboflavin in fluid milk packaged in glass containers and subjected to sunlight was one of the first nutrient losses in food products documented by scientific study. More recent studies have documented riboflavin loss in translucent containers in refrigerated dairy cases leading to the use of light barriers in blow-molded polyethylene containers.[35] Even in dry products, light exposure can lead to riboflavin loss. Enriched pasta lost up to 80% of the riboflavin within 12 weeks under fluorescent light.[36]

Various studies have defined riboflavin stability during common cooking or food processing operations. Prodarov et al.[37] showed that up to 70% of the riboflavin in the chick-peas is lost by soaking in sodium bicarbonate solution followed by cooking (Figure 7.6). Likewise, riboflavin degradation in spinach during various types of cooking processes follows first order kinetics.[38] Fermentation can increase riboflavin content.[37,39] Batifoulier et al.[39] reported 30% enrichment in whole wheat bread owing to the yeast fermentation.

In a more recent study, Batifoulier et al.[40] found that riboflavin concentration was twofold higher in bread than in the white flour used to produce the bread. The extreme lability under even dim light conditions dictates that the analysts protect riboflavin preparations under all phases of analysis. Darkness and use of red light and low-actinic glassware as well as temperature control can minimize riboflavin loss during analysis.

Stability in plasma can be problematic. Akimoto et al.[41] studied the stability of FAD in rat and human plasma and reported that low-intensity light rapidly converted FAD to riboflavin

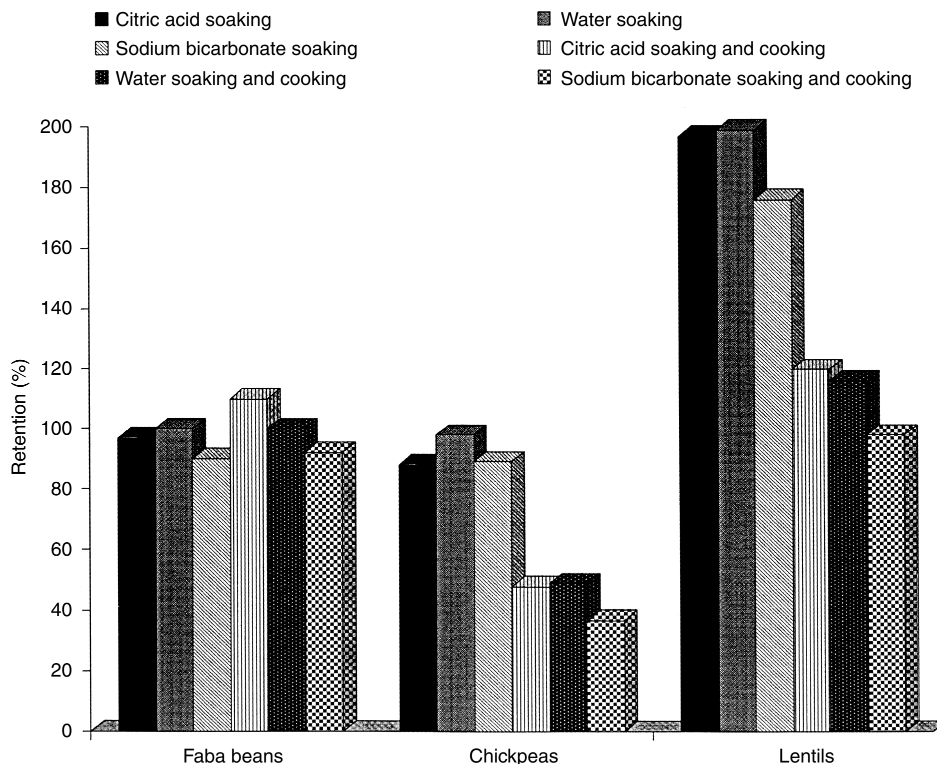

Figure 7.6 Effect of processing on the riboflavin content of legumes. (Reproduced from Prodanov, M., Sierra, I., and Vidal-Valverde, C., Influence of soaking and cooking on the thiamin, riboflavin, and niacin contents of legumes, *Food Chem.*, 84, 271, 2004. With permission.)

and FMN. Pretreatment of plasma with EDTA inhibited the conversion. These authors recommended strict light control, temperature, and addition of EDTA to prevent hydrolysis of FAD in plasma samples.

7.3 Methods

Rat and chick assays were developed for the analysis of riboflavin in the early 1930s. The animal assays were mostly replaced in the next decade by microbiological and fluorometric procedures followed by high performance liquid chromatography (HPLC) techniques in the 1970s. Useful reviews on methodology for riboflavin assay include Russel and Vanderslice,[7] Ball,[15] Nielsen,[11] Eitenmiller and Landen,[14] and Ottaway.[18] Regulatory and other standard methods are summarized in Table 7.4.[42–61]

7.3.1 Classical approaches to analysis of riboflavin

7.3.1.1 Fluorometric

7.3.1.1.1 Direct fluorometry. Association of Official Analytical Chemists (AOAC) International methods[44] are based on the measurement of the native fluorescence of riboflavin. These methods include

1. AOAC Official Method 970.65, Riboflavin (Vitamin B_2) in Foods and Vitamin Preparations—Fluorometric Method, AOAC Official Methods of Analysis, 45.1.08.

Table 7.4 Regulatory and Handbook Methods for the Analysis of Riboflavin

Source	Form	Method and application	Approach	Most current cross-reference
***U.S. Pharmacopeia National Formulary*, 2006, USP 29/NF 24, Dietary Supplements Official Monographs**[42]				
1. Pages 2392–2393, 2394, 2396, 2399, 2404, 2419–2421	Riboflavin	Riboflavin in oil and water-soluble capsules/tablets w/wo minerals	Method 1—LC 280 nm Method 2—LC 254 nm Method 3—LC 270 nm	None
2. Pages 2428–2430, 2432–2433, 2435–2436	Riboflavin	Riboflavin in water-soluble capsules/tablets w/wo minerals	Method 1—LC 280 nm Method 2—LC 254 nm Method 3—LC 270 nm	None
3. Page 1913	Riboflavin	Riboflavin (NLT 98.0%, NMT 102.0%)	Fluorescence Ex $\lambda = 444$ Em $\lambda = 530$	
4. Pages 1913–1914	Riboflavin	Riboflavin tablets/injection	Fluorescence Ex $\lambda = 440$ Em $\lambda = 530$	None
5. Pages 1914–1915	Riboflavin 5′-phosphate sodium and other phosphate esters	Riboflavin 5′-phosphate sodium	LC Fluorescence Ex $\lambda = 470$ nm Em $\lambda = 530$ nm	None
***British Pharmacopeia*, 2007**[43]				
1. Pages 1804–1805	Riboflavin	Riboflavin	Spectrophotometric 444 nm	None
2. Pages 1805–1807	Riboflavin 5′-phosphate sodium salt	Riboflavin sodium phosphate	Spectrophotometric 444 nm	None
3. Pages 2989–2990	Riboflavin sodium phosphate	Vitamins B and C injection 446 nm	Spectrophotometric None	

Continued

Table 7.4 (Continued)

Source	Form	Method and application	Approach	Most current cross-reference
AOAC International Official Methods of Analysis, 18th Ed., 2005[44]				
1. 45.2.01	Riboflavin	AOAC Official Method 960.46 Riboflavin, Microbiological Assays	Microbiological	*J. Assoc. Off. Anal. Chem.*, 42, 529, 1959[45]
2. 45.1.08	Riboflavin	AOAC Official Method 970.65 Riboflavin in Foods and Vitamin Preparations	Fluorescence Ex $\lambda = 440$ Em $\lambda = 565$	*J. Assoc. Off. Anal. Chem.*, 53, 542, 1970[46]
3. 45.1.09	Riboflavin	AOAC Official Method 981.15	Fluorescence Ex $\lambda = 430$ Em $\lambda = 557$	*J. Agric. Food Chem.*, 23, 815, 1975;[64] *J. Assoc. Off. Anal. Chem.*, 62, 1041, 1979[65]
4. 50.1.07	Riboflavin	AOAC Official Method 985.31 Riboflavin in Ready-To-Feed Milk-Based Infant Formulas	Fluorescence Ex $\lambda = 440$ Em $\lambda = 565$	*J. Assoc. Off. Anal. Chem.*, 68, 514, 1985[47]
5. 45.2.06	Riboflavin	AOAC Official Method 940.33 Riboflavin in Vitamin Preparations	Microbiological	*J. Assoc. Off. Anal. Chem.*, 42, 529, 1959[45]
American Association of Cereal Chemists, Approved Methods, vol. 2, 2000[48]				
1. AACC 86-70	Riboflavin	Riboflavin in whole-grain, grits, meal, and puffed cereal, farina and bread	Fluorescence Automated Ex $\lambda = 436$ Em $\lambda = 570$	*Cereal Sci. Today*, 7, 198, 1962;[49] Anal. Chem., 19, 243, 1947[50]

2. AACC 86-72	Riboflavin	Riboflavin in whole-grain, grits, meal, flaked and puffed cereal, farina and bread	Microbiological	*Ind. Eng. Chem.*, 14, 271, 1942;[51] 17, 176, 1945[52]
3. AACC 86-73	Riboflavin	Riboflavin in whole-grain, grits, meal, flaked and puffed cereal, farina and bread	Fluorescence Automated Ex λ = 436	*J. Agric. Food Chem.*, 23, 815, 1975[53]
Food Chemicals Codex, 5th Ed., 2004[54]				
1. pages 338-339	Riboflavin	Riboflavin (NLT 98.0%, NMT 102.0%)	Fluorescence Ex λ = 440 Em λ = 530	None
2. page 339-341	Riboflavin 5′-phosphate sodium	Riboflavin 5′-phosphate sodium (NLT equivalent of 73.0% of Riboflavin, NMT equivalent of 79.0% of Riboflavin)	Fluorescence Ex λ = 440 Em λ = 530	None
Methods for Determination of Vitamins in Foods Recommended by COST 91[55]				
	Riboflavin	Tentative methods, foods	LC Fluorescence Ex λ = 453 Em λ = 521	Anal. Chem., 130, 359, 1983[56]
European Committee for Standardization, 2003[57]				
EN14152	Riboflavin	Foods	LC Fluorescence Ex λ = 468 Em λ = 520	*Food Chem.*, 57, 101, 1996;[58] *J. Micronutr. Anal.*, 3, 251, 1987;[59] *Food Chem.*, 56, 81, 1996;[60] 71, 129, 2000[61]

2. AOAC Official Method 981.15, Riboflavin in Foods and Vitamin Preparations—Automated Method, AOAC Official Methods of Analysis, 45.1.09.
3. AOAC Official Method 985.31, Rib oflavin in Ready-to-Feed Milk-Based Infant Formula—Fluorometric Method, AOAC Official Methods of Analysis, 50.1.07.

These methods are closely related in principle and will be discussed to explain the chemistry, method development, and application aspects.

Manual Procedure

AOAC Official Method 970.65 has been the primary method used to determine published riboflavin content of the food supply. It remains an important procedure for analysis of riboflavin.[62,63] The Atlanta Center for Nutrient Analysis, Southeastern Laboratory Food and Drug Administration uses this method for routine nutritional label compliance analysis. Method 970.65 measures total riboflavin after acid hydrolysis of FMN and FAD to free riboflavin. Hydrolysis is completed by autoclaving in 0.1 N HCl for 30 min. Specific parameters are provided in the AOAC Official Methods of Analysis[44] for preparation of dry and semidry samples, those containing basic materials, liquids, concentrates, premixes, and multivitamins. Proteins are removed by adjusting the pH to 4.5 and filtering or centrifuging to clarify the extract. pH is sequentially increased to 6.8 to check that no further precipitation occurs.

Interfering fluorescent substances are destroyed by acidifying the extract with glacial acetic acid and oxidation with potassium permanganate. After 2 min of oxidation, excess permanganate is destroyed with the addition of hydrogen peroxide. Hydrogen peroxide decolorizes the solution. If MnO_2 precipitate is noted at this point, the solution should be filtered or centrifuged. The oxidation step does not affect riboflavin, but is essential to limiting fluorescence as much as possible to riboflavin. A long standing criticism of the general procedure is that an overestimation of the true riboflavin content can occur through nonspecific fluorescence.

Fluorescence measurements (Em $\lambda = 565$, Ex $\lambda = 440$) include sample solutions containing added riboflavin as an internal standard (spike) and on sample solutions containing water in place of the spike solution. After initial measurement of the sample fluorescence, sodium hydrosulfite is added to sample tubes and fluorescence is measured. The sodium hydrosulfite converts riboflavin to the nonfluorescent, reduced leuco form to provide a blank to correct for remaining fluorescence (not from riboflavin) after the addition of permanganate oxidation. The AOAC International procedure notes that addition of sodium hydrosulfite in excess of 20 mg per tube can reduce interfering fluorescing materials, leading to inaccuracies in the assay.

Modifications of AOAC International Method 970.65

Method 970.65 was modified for flow-injection analysis to reduce manual error and increase laboratory output. AOAC Method 981.15 is a semiautomated procedure based on Method 970.65. Original studies were completed by Egberg and Potter[64] and Egberg.[65] The procedure used the Technicon AutoAnalyzer II system. Modifications to the manual method include the use of sodium bisulfite to reduce excess permanganate in place of hydrogen peroxide to eliminate oxygen bubble formation within the system. Blanks are run by replacing the sodium acetate diluent added in front of the fluorometer with sodium hydrosulfite solution. The samples are then pumped through the system a second time for the blank determination. To prevent precipitation and build up of MnO_2 in the coil, where the excess permanganate is reduced, metaphosphoric acid is introduced as a manganese sequestering ligand. Egberg and Potter[64] obtained a correlation coefficient of 0.9869 with an overall standard error of 0.23 mg 100 g^{-1} when the manual and semiautomated methods were compared

on 61 different foods. Method 981.15 gives comparable results to the microbiological assay by Lactobacillus casei ssp. rhamnosus.[13]

Semiautomated procedures based on the Technicon Auto Analyzer were modified to a simpler flow-injection analysis system.[63] Lack of defatting steps in the AOAC International methods led to low recoveries for some matrices. This study also showed that raw liver could rapidly decrease spiked riboflavin if the addition was completed before autoclaving. Indigenous enzymes were, therefore, indicated to act rapidly to degrade riboflavin. The authors concluded that AOAC International methods were not suitable for samples containing active enzyme systems.[63] This, again, draws attention to the need for the analyst to pay particular attention to stability factors early in the extraction stage for the analysis of all vitamins.

7.3.1.1.2 Indirect fluorometry. Excellent discussions exist in the older literature on the use of lumiflavin for the indirect quantitation of riboflavin.[66,67] Riboflavin is converted to lumiflavin when irradiated under alkaline conditions (Figure 7.4). The reaction adds specificity since other fluorescing compounds in the sample extract are not converted to lumiflavin. Further specificity is added by extraction of the lumichrome into chloroform. Lumiflavin increases sensitivity since it fluoresces more strongly than native riboflavin (Ex $\lambda = 450$, Em $\lambda = 513$). Chloroform extraction of lumiflavin eliminates naturally occurring fluorescent materials from the sample extract that are not soluble in chloroform. Even though these advantages exist for the lumiflavin procedure, the method has seen little use.[15] The strong fluorescence and specificity of lumiflavin can be applied to LC methodology, eliminating the need for extract cleanup. Lumiflavin was used for the LC analysis of riboflavin in meat and meat products with detection limits as low as 0.05 ng per injection.[22] Addition of riboflavin standard to the sample as an internal reference standard is recommended to compensate for possible intensity differences in irradiation of the sample extract and the reference riboflavin solution.[66] Extractions of natural products might require amylase or protease treatment to obtain optimal extraction efficiency.

7.3.1.2 Microbiological

Lactobacillus casei ssp. rhamnosus ATCC 7469 is considered to be the best and most-often used microorganism for riboflavin assay. Since the *L. casei* response to FMN and FAD differs from the growth promotion by riboflavin, all methods based on *L. casei* are for total riboflavin. Procedures incorporate an acid hydrolysis step to convert FMN and FAD to free riboflavin. Other microorganisms including *Leuconostoc mesenteroides*,[67] *Tetrahymena pyriformis*,[68] and *Enterococcus faecalis*[15,69] have riboflavin growth requirements but have not been extensively used. *L. casei* growth is affected by common biological sample constituents including starch, protein degradation products, and free fatty acids. Free fatty acids can stimulate or inhibit the growth response, and fat should be extracted with ether or hexane before the acid hydrolysis step. The fat extraction step should be considered routine unless the analyst verifies that the fat content of the sample is inconsequential to the assay results. Proteins are removed through precipitation at pH 4.5. Ball[15] points out that *E. faecalis* is not affected as extensively by matrix effects compared to *L. casei*, and that it could be used in place of *L. casei*. However, lack of commercial media for the *E. faecalis* assay makes its use more difficult.

AOAC International Method 940.33, "Riboflavin (Vitamin B_2) in Vitamin Preparations, Microbiological Methods (960.46)," Chapter 45.2.06, is approved only for vitamin preparations. However, because of the broad application of *L. casei* to food analysis, the AOAC Task Force on Methods for Nutrient Labeling recommended collaboration of the assay for all food matrices.[70] The procedure was not collaborated. In the procedure, FMN and FAD are converted to free riboflavin by hydrolysis with 0.1 N HCl (121°C for 30 min). The hydrolyzed extracts are adjusted to pH 4.5, precipitated protein is filtered or centrifuged from the

extract and the pH is adjusted to 6.8. Digestion with amylolytic or proteolytic enzymes is not required in Method 940.33. However, enzyme digestions can be highly beneficial for efficient extraction of riboflavin from high starch or high protein matrices as well as ensuring the complete conversion of FMN and FAD to riboflavin.

Combined extractions for thiamin and riboflavin analysis have been commonly used for food analysis.[71,72] The European Measurement and Testing Program reported an optimal combined extraction procedure for thiamin and riboflavin suitable for either microbiological or LC analysis.[58,73] Details of this extraction are given in Chapter 14.

7.3.2 Advances in the analysis of riboflavin

7.3.2.1 Spectroscopic methods

The spectral properties of riboflavin provide an ideal analyte for application of advanced spectroscopic methods based on fluorescence spectrometry. In addition, flow injection combined with chemiluminescence provides a good analytical approach. Various recent methods based on fluorescence and chemiluminescence are summarized in Table 7.5.[74–84]

7.3.2.2 Capillary electrophoresis

Capillary electrophoretic methods have been effectively utilized for analysis of riboflavin, primarily from pharmaceutical products with high vitamin levels. The procedures are adaptable to multianalyte analysis. Owing to the strong fluorescence of riboflavin and its metabolites, laser-induced fluorescence (LIF) detection has become the primary detection mode coupled with the resolution power of capillary electrophoresis (CE). Fluorescence detection provides greatly increased sensitivity and selectivity compared to absorbance detectors. Capillary electrophoresis methods for riboflavin, FMN, and FAD are summarized in Table 7.6.[85–98]

7.3.2.3 Liquid chromatography

Reviews on LC methods for analysis of riboflavin, FMN, and FAD include Finglas and Faulks,[59] Van Niekerk,[99] Russell and Vanderslice,[7] Nielsen,[11] Ottaway,[18] and Ball.[15] Russell and Vanderslice[7] categorized LC methods for riboflavin and its coenzymes as follows:

1. Total riboflavin analysis using C_{18} columns and fluorescence detection
2. Total riboflavin analysis using C_{18} columns and UV detection
3. Total riboflavin analysis using silica and amino columns with either fluorescence or UV detection
4 Individual flavin analysis excluding the simultaneous determination of riboflavin, FMN, and FAD
5. Simultaneous analysis of riboflavin, FMN, and FAD.

To broaden this classification, LC methods for the concurrent or simultaneous analysis of other water-soluble vitamins in addition to total riboflavin should be added. In more recent published methods for food analysis, thiamin and riboflavin are frequently analyzed together. We define concurrent methods as those that utilize the same sample extract with different chromatography systems; whereas, simultaneous procedures quantitate the two vitamins from the same LC injection. LC methods for riboflavin (total) are summarized in Table 7.7.[35,41,62,100–106,107–111,112–121] Concurrent and simultaneous methods including riboflavin and thiamin are discussed in Chapter 14.

7.3.2.3.1 *Extraction procedures for analysis of riboflavin by LC.*

Summaries of extraction procedures for riboflavin used for LC analysis are provided in Table 7.7. Total riboflavin

Table 7.5 Selected Spectroscopic Methods for Riboflavin Analysis—Fluorescence and Chemiluminescence

Matrix	Description	Accuracy/precision	References
Fluorescence			
Pharmaceuticals	Synchronous fluorescence in the presence of bis-2-ethylhexyl sulfosuccinate sodium salt micelles was used for the simultaneous assay of riboflavin, pyridoxine and thiamin	%RSD <14 DL—9.0 μg L^{-1}	*Anal. Chim. Acta*, 434, 193, 2001[74]
Beer	Quenching of riboflavin fluorescence by aporiboflavin-binding protein from egg white provided a quantitative method	DL—10 mM	*J. Agric. Food Chem.*, 50, 1548, 2002[75]
Yogurt	Fluorescence landscapes (Ex λ from 270–550 nm, Em λ from 310–590) obtained from front-face fluorescence were combined with chemometrics to assay riboflavin	Prediction error—0.092 μg g^{-1}	*J. Dairy Sci.*, 86, 2508, 2003[76]
Cereal flours	Front-face emission spectra of flour provided a quantitative riboflavin assay	%RSD ≤1.6	*J. Agric. Food Chem.*, 51, 2888, 2003[77]
Salmonid eggs	Solid phase extraction with end-capped C18 reversed phase columns provided extracts sufficiently clean for direct fluorescence assay of riboflavin. The assay simultaneously assayed thiamin.	—	*J. Aquatic Animal Health*, 17, 95, 2005[78]
Chemiluminescence			
Pharmaceuticals	FIA Inhibition of the intensity of chemiluminescence from luminol-$K_3Fe(CN)_6$ by riboflavin, throughput = 30 h^{-1}	%RSD <2.2 %Recovery—98–102 DL—0.01 μg mL^{-1}	*Anal. Lett.*, 33, 2767, 2000[79]
Pharmaceuticals	FIA Enhancement of the light induced chemiluminescence reaction of luminol oxidized by periodate	DL—1 × 108 mol L^{-1}	*Anal. Lett.*, 33, 3285, 2000[80]
Pharmaceuticals, urine	FIA Enhancement of the light induced chemiluminescence reaction of luminol with periodate, throughput = 120 h^{-1}	%RSD <3.0 DL—0.02 ng mL^{-1}	*Analyst*, 126, 1393, 2001[81]
Pharmaceuticals	Electrogenerated Chemiluminescence Enhancement of the electrogenerated chemiluminescence of lucigenin by riboflavin at a platinum electrode	%RSD – 4.0 DL—8.2×10^{-9} g mL^{-1}	*Anal. Sci.*, 18, 819, 2002[82]
Pharmaceuticals	FIA Enhancement of the chemiluminescence produced during the oxidation of N-bromosuccinimide (NBS) and N-cholorsuccinimide (NCS) in alkaline media by riboflavin	DL (ng mL^{-1}) NBS—7.5 NCS—3.5	*Luminescence*, 20, 170, 2005[83]
Biosensor-based			
Milk	Measurement of the free riboflavin binding protein after formation of riboflavin complex by surface plasmon resonance with on-chip measurement	DL—70 μg L^{-1} QL—234 μg L^{-1}	*Anal. Chem.*, 76, 137, 2004[84]

Table 7.6 Application of Capillary Electrophoresis to Riboflavin Analysis

Matrix	Description	Accuracy/precision	References
Pharmaceuticals B_1, B_2, B_6	CZE 51 cm × 75 μm, 0.02 M sodium phosphate, pH 9.0, 6.0 kV, 254 nm	%RSD—2.1–6.3 %	*J. Chromatogr*., 636, 133, 1993[85]
Pharmaceuticals B_1, B_2, B_6, B_{12}	CZE, MECC 5.7 cm × 75 μm, 0.02 M borate—0.02 M sodium dihydrogen phosphate (CZE), add 0.1 M SDS for MECC, 20 kV, 214 nm	%Recovery—99.7 %RSD—2.2	*J. Chromatogr. A*, 670, 209, 1994[86]
Human plasma B_1, FMN, FAD	CZE, MECC 37.5 cm × 75 μm, 100 mmol L^{-1} sodium borate, pH 7.9, with 50 mmol L^{-1} SDS, 100 mL L^{-1} methanol and 20 mL L^{-1} *N*-methylformamide, 24 kV, LIF, Ex λ = 488, Em λ = 530	%CV (within-day) B_2—6–9 FMN—4–9 FAD—5–7 %Recovery—90–103	*Clin. Chem*., 45, 862, 1999[87]
Tissues B_2, FMN, FAD	CZE 57 cm × 75 μm, 50 mM borate, pH 8.5, 14 kV, LIF, Ex λ = 488, Em λ = 530	DL ≤ 0.23 mol L^{-1}	*Electrophoresis*, 22, 1170, 2001[88]
Wine, milk, yogurt, eggs, other foods B_2, FMN, FAD	CZE 84 cm × 75 μm, 30 mM phosphate, pH 9.8, 30 kV, LIF, Ex λ = 442, Em λ = 515	%Recovery Wine > 90 DL (a mol) B2—50 FMN—350 FAD—300	*J. Agric. Food Chem*., 50, 6643, 2002;[89] *J. Chromatogr. A*, 968, 229, 2002;[90] *Food Chem*., 82, 309, 2003;[91] www.legceurope.com[92]

FMN, FAD Model systems, biologicals	Dynamic pH Junction-Sweeping CE or MECC 57 cm × 75 μm, 140 mM borate, pH 8.5–10.0, add 100 mM SDS for MECC, LIF, Ex λ = 488, Em λ = 520	DL—4.0 pM	*Anal. Chem.*, 74, 3736, 2002;[93] *Anal. Biochem.*, 313, 89, 2003[94]
Recombinant flavin-binding domains B_2, FMN, FAD	Microchip CE with LIF, Ex λ = 488, Em λ = 515, 40 mM phosphate buffered saline, pH 8.0–10.0	DL (mM) B_2—34 FMN—127 FAD—201	*J. Chromatogr. A*, 1021, 201, 2003[95]
Urine, beer B_2	MECC or Stacking-MECC 10 mM sodium tetraborate with 80 mM SDS	DL (ng mL^{-1})	*J. Chromatogr. B*, 785, 39, 2003;[96] *Talanta*, 64, 970, 2004[97]
	Dynamic pH Junction 100 mM sodium tetraborate, pH 8.2	MEEC—480 Stacking MEEC—20	
	Dynamic pH Junction-Sweeping 100 mM sodium tetraborate—100 nM SDS, Detection—Blue light emitting diode	Dynamic junction—1	
Biologicals, food, beverages B_2, FMN, FAD	Microchip CE with LIF, Ex λ = 450, Em λ = 520, 20 mM phosphate, pH 9.2	DL—0.15–1.0 μg L^{-1}	*J. Chromatogr. A*, 1027, 223, 2004[98]

Table 7.7 LC Methods for the Analysis of Riboflavin in Foods, Feed, Pharmaceuticals, and Biologicals

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods					
Milk, dairy products/ riboflavin	Milk – pass through C_{18} Sep-Pak, elute riboflav in w/ 0.02 M acetate buffer, pH 4.0:MeOH (1:1). Dairy products – blend w/0.02 M acetate buffer, pH 4.0, pass through C_{18} Sep-Pak	Bio-Sil ODS-5S C_{18}, 25 cm × 4 mm Mobile phase—isocratic Water:MeOH:HAC (65:35:0.1) Flow rate—1 mL min^{-1}	270 nm	DL (on column)—10 ng %CV—1.6–5.4 %Recovery—94.5–100.7	*J. AOAC*, 68, 693, 1985[108]
Cheese/riboflavin	Homogenize in water: MeOH (2:1), acidify w/HAC, centrifuge	LiChrosorb RP_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase – isocratic Water:MeCN (80:20) Flow rate—1 mL min^{-1}	446 nm	DL (on column)—2.5 ng %CV—2.1–6.5 %Recovery—90.2	*J. Food Sci.*, 51, 857, 1986[109]
Milk/riboflavin	Mix 2 mL 10% lead acetate w/20 mL milk, filter	LiChrosorb C_{18}, 10 µm, 25 cm × 4.6 mm Mobile phase – isocratic Water:MeOH:HAC (50:49:1) Flow rate—1.5 mL min^{-1}	Fluorescence Filter fluorometer	—	*J. Food Sci.*, 53, 436, 1988[35]
Various foods/riboflavin	Homogenize, add 0.2 M H_2SO_4, autoclave, 20 min. Adjust pH to 4.5 w/2.5 M acetate buffer. Digest w/Claradiastase or Takadiastase, 45°C. Dilute, filter Clean-up: Sep-Pak C_{18}	Spherisorb S5 ODS2, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic Water:MeOH (65:35) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 445 Em λ = 525	DL (on column)—20 ng %CV—3.4–6.2 %Recovery—93–108	*J. Micronutri. Anal.*, 8, 199, 1990[110]

Various foods/riboflavin	Add 0.1 N HCl (30 mL) and 6 N HCl (1 mL) to 5 – 10 g sample, autoclave, 15 min. Adjust pH to 4.0–4.5, digest w/Takadiastase, 48°C, 3 h. Filter and dilute. Clean-up: Florisil followed by Sep-Pak C_{18}	μBondpak C_{18}, 10 μm, 30 cm × 3.9 mm Mobile phase–isocratic Water:MeOH:HAC (67:32:1) containing 5 mM Na Hexsulfonate or Water: MeOH:HAC (68:31:0.5) containing 5 mM Na heptane sulfonate and 5 mM hexsulfonate (25:75)	254 nm	DL (on column)—0.4 ng %Recovery—98	*J. Liq. Chromatogr.*, 13, 2089, 1990[111]
Dairy products/ riboflavin, FMN, FAD	Add formic acid – urea (6% formic acid containing 2 M urea). Homogenize or mix. Centrifuge to remove fat. Add sorboflavin (IS). Clean – up: Silica gel C_{18}	Supelco LC18, 3 μm, 7.5 cm × 4.6 mm Mobile phase – isocratic 100 mM KH_2PO_4:MeCN (86:14), pH 2.9 Flow rate – 1 mL min^{-1}	Fluorescence Ex λ = 450 Em λ = 530	DL (nmol L^{-1}) FMN—2.5 B2—2.5 FAD—3 %CV—6.7 %Recovery—95–101	*J. Chromatogr.*, 511, 359, 1990[102]
Various foods/riboflavin, FMN, FAD	Add MeOH (9.0 mL) and CH_2Cl_2 (10 mL) per 0.5 – 4.0 g sample. Add 7-ethyl-8-methyl-riboflavin (IS). Homogenize. Add 100 mM citrate-phosphate buffer, pH 5.5 containing sodium azide, homogenize, centrifuge, and filter	2 PLRP-S 100 Å in series, 5 μm 25 cm × 4.6 mm and 15 cm × 4.6 mm Mobile phase – gradient (MeCN: 0.1% sodium azide in 10 mM citrate-phosphate buffer, pH 5.5)	Fluorescence Ex λ = 450 Em λ = 522	%Recovery—96–113	*Food Chem.*, 43, 151, 1992;[62] *Food Chem.*, 63, 125, 1998[101]

Continued

Table 7.7 (Continued)

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Milk, nondairy imitation milk/ Riboflavin	Add 10 lead acetate, pH 3.2, filter	Spherisorb ODS 5 μm, 15 cm × 3.9 mm Mobile phase–isocratic Water-HAC: MeOH (70:30) Water-HAC: 1.3 mL HAC in 1 L water Flow rate–0.6 mL min^{-1}	270 nm	%Recovery–92.2 ± 1.2	*Food Chem.*, 49, 203, 1994[107]
Food/Riboflavin, FMN, FAD	Suspend sample (2-20 g) in 19 mL MeOH-CH_2Cl_2 (9:10, v/v), shake for 60 s. Add 9 mL 0.1 M ammonium hydrogencarbonate, pH 7.0 and shake another 60 s, centrifuge	Symmetry C18 5 μm, 15 cm × 3.9 mm Mobile phase–gradient MeOH–0.05 M NH4OAC, pH 6.0 Flow rate–gradient	Fluorescence Ex λ = 450 Em λ = 530	DL (ng L^{-1}) RF–1 FMN–1 FAD–6 %CV < 2.82 %Recovery >95	*J. Chromatogr. A*, 822, 59, 1998;[103] 881, 285, 2000;[105] *J. Agric. Food Chem.*, 47, 3197, 1999[104]
Foods/Riboflavin, FMN, FAD	Add 10 mL MeCN to 1–2 g solid sample, homogenize 10 min. Add 10 mL 10 mM phosphate buffer, pH 5, homogenize and centrifuge. Dilute supernatant to 25 mL w/phosphate buffer, filter and inject. Filter liquid sample and inject	Discovery RP-Amide C_{16}, 5 μm, 15 cm × 4.6 mm Mobile phase–isocratic MeCN:10 mM KH_2PO_4 (pH 5) (10:90) Flow rate–1 mL min^{-1}	Fluorescence Ex λ = 270 Em λ = 516	DL (ng) RF–0.03 FMN–0.05 FAD–0.24	*J. Agric. Food Chem.*, 52, 1789, 2004[106]

Biologicals					
Blood/Riboflavin, FMN, FAD	Add 10% TCA, hold at 4°C, 30 min. Add NaOAC buffer, centrifuge	Hypersil ODS 5 μm, 15 cm × 4.6 mm Mobile phase–isocratic 0.3 M KH_2PO_4:MeOH (83.3:16.7) pH 2.9 Flow rate–2 mL min^{-1}	Fluorescence Ex λ = 470 Em λ = 525	DL (nmol L^{-1}) B_2–10 FAD–20 FMN–15 QL–240 nmol L^{-1} for FAD %Recovery–94	*J. Chromatogr.*, 228, 311, 1982[113]
Serum, wine/Riboflavin	Add isoriboflavin (IS) and add TCA (100 g L^{-1}). Centrifuge. Clean-up: Sep-Pak C18 for serum	ROSIL C18 HL 5 μm, 15 cm × 3.2 mm Mobile phase–isocratic Water:HAC:MeOH (63.7:0.1:36.3) Flow rate–0.75 mL min^{-1}	Fluorescence Ex λ = 450 Em λ = 530	DL—10 μg L^{-1} for serum %CV—2.3–4.9	*Clin. Chem.*, 318, 1371, 1985[114]
Urine/Riboflavin	Add HAC and toluene. Saturate with $(NH_4)2SO_4$. Centrifuge. Add 80% aqueous phenol. Centrifuge. Add water. Extract with water saturated Et_2O	μBondaPak C18, 5 μm, 25 cm × 4.6 mm Mobile phase–gradient 5 mM NH4OAC, pH 6.0 to 21% MeOH to 50% MeOH to 70% MeOH over 21 min Flow rate–1 mL min^{-1}	Fluorescence Ex λ = 305–365 Em λ = 475–650	—	*J. Nutr.*, 117, 468, 1987;[115] *Am. J. Clin. Nutr.*, 46, 830, 1987[116]
Plasma, urine/Riboflavin, FMN, FAD, degradation product	Plasma–add MeCN to plasma (1:1). Centrifuge, extract w/ $CHCl_3$. Assay aqueous phase. Urine–inject directly or dilute w/water	PRP-1 10 μm, 25 cm × 4.6 mm Mobile phase–isocratic Water: 10% trifluoroacetic acid:MeCN (84:1.5:14), pH 1.8	Fluorescence Ex λ = 470 Em λ = 525	DL–1–5 ng mL^{-1} %Recovery >84	*J. Chromatogr.*, 423, 105, 1987[100]

Continued

Table 7.7 (Continued)

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Plasma/Riboflavin, FMN, FAD	Add 1 mL 15 mM magnesium acetate to 1 mL plasma, incubate at 65°C, 15 min. Add 0.5 mL 10% TCA to precipitate protein, centrifuge at 4, 10 min. Keep supernatant, add 1 mL 15 mM magnesium acetate to the pellet, centrifuge. Combine two supernatants. Clean-up: C18 Sep-Pack cartridge	C_{18} RP column 5 μm, 25 cm × 4 mm Mobile phase – isocratic Phosphate-magnesium acetate buffer (pH 3.4): MeCN (85:15) Flow rate: 1 mL min^{-1}, IS-galactoflavin	Fluorescence Ex λ = 445 Em λ = 530	%Recovery > 97 %CV – 2.9–5.8	*J. Chromatogr., B*, 739, 219, 2000;[112] *Biol. Pharm. Bull.*, 29, 1779, 2006[41]
Human plasma/ riboflavin, FAD, FMN	Add TCA. Centrifuge. Incubate supernatant, 85°C, 10 min. Inject directly	Gynkotek RP 18, 10 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:5 μM NH4OAC (35:65) Flow rate—1.8 mL min^{-1}	Fluorescence Ex λ = 450 Em λ = 520	DL (nmol L^{-1}) Riboflavin—3.0 FAD, FMN—9.0 %Recovery—82–94	*Ann. Nutr. Metab.*, 39, 224, 1995[117]
Urine/riboflavin	Direct injection	YMC-PackPro C_{18}, 5 μm, 15 cm × 4.6 mm Mobile phase—isocratic MeOH:water (50:50) Flow rate—0.7 mL min^{-1}	Fluorescence Ex λ = 450 Em λ = 530	$\%CV_{Intra}$—0.7–3.3 $\%CV_{Inter}$—2.9–9.0	*J. Chromatogr. B*, 820, 147, 2005[118]

Cell/Riboflavin, FMN, FAD	Extract cell w/ 2 mL ice-cooled MeOH. Centrifuge. Add 1.6 mL chloroform and 0.64 mL deionized water to the supernatants, mix and centrifuge. Filter upper layer and evaporate the filtrate, reconstitute in 0.5 mL deionized water	Capillary LC system–Monolithic silica-ODS column, 50 cm × 0.2 mm Mobile phase—gradient MeOH:30 mM phosphate buffer (pH 3) Flow rate—2 μL min^{-1}	265 nm	LOD (ng mL^{-1}) RF—12 FMN—28 FAD—28 %RSD—1.5–2.9	*J. Chromatogr. A*, 1053, 71, 2004[119]
LC-MS					
Blood products/ riboflavin, lumichrome, lumiflavin	Spike platelet concentrate with 50 μM riboflavin, illuminate. Centrifuge. Dilute supernatant 1:50 with 0.9% saline	Zorbax 80°A SBCN, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient Water:MeOH (77:23) to 100% MeOH over 18 min Flow rate—1 mL min^{-1}	Fluorescence 1st 14.5 min Ex λ = 268 Em λ = 525 Followed by Ex λ = 260 Em λ = 470 LC-MS/MS-ESI Negative ion mode	DL (μM) Riboflavin–0.0006 Lumichrome–0.012	*Photochem. Photobiol.*, 80, 609, 2004[120]
Semisynthesis product/ Riboflavin, lumiflavin	Information not provided	Zorbax SB-C18, 5 cm, 15 cm × 4.6 mm Mobile phase—isocratic MeOH:water (75:25) Flow rate—1.0 mL min^{-1}	224 mm LC-MS-ESI Negative ion mode	%Recovery—98-100 %RSD—0.5–1.5	*J. Chromatogr. Sci.*, 44, 552, 2006[121]

analysis is initiated with acid hydrolysis by HCl or H_2SO_4 to convert coenzyme forms into free riboflavin. These procedures either duplicate or closely follow procedures recommended for the chemical or microbiological assay procedures. Solid phase cleanup procedures are often used before injection. Supports for extract cleanup include C_{18} or Florisil. Extraction procedures for urine and serum commonly start with the addition of trichloroacetic acid (TCA), ammonium sulfate, or metaphosphoric acid to the sample for deproteinization. When TCA is used, excess TCA must be removed with water-saturated diethyl ether. These procedures do not incorporate acid hydrolysis and, therefore, allow quantitation of FAD and FMN in addition to free riboflavin. Lopez-Anaya and Mayersohn[100] precipitated proteins in urine with acetonitrile followed by shaking with chloroform and injected the extract directly for analysis of riboflavin, FMN, and FAD in urine and plasma.

Specific methods for quantitation of riboflavin and its coenzymes in foods include those by Russell and Vanderslice,[62] Russell et al.[101] and Bilic and Sieber.[102] Russell and Vanderslice[62] and Russell et al.[101] extracted the flavins with methanol and dichloromethane followed by partitioning with 100 mM citrate phosphate buffer, pH 5.5. Bilic and Sieber[102] extracted dairy products with 6% formic acid containing 2 M urea followed by C_{18} solid phase cleanup. Both procedures used internal standards and proved useful for accurate quantitation of the native riboflavin forms in foods. More recent methods by Gliszczymska and Koziolowa[103–105] modified the Russell and Vanderslice procedure, [62] replacing citrate–phosphate buffering solution with ammonium hydrogen carbonate. The extraction was applied to the assay of riboflavin, FMN, and FAD in yeast, yogurt, and other foods. Viñas et al.[106] used a simple acetonitrile and phosphate buffer extraction to assay flavins in foods including infant formulas, beer, juices, and honey.

Usually, acid digestion with autoclaving completely liberates riboflavin from bound forms. However, difficult matrices such as those high in starch or protein can be more effectively extracted through use of amylolytic or proteolytic enzymes.[7] Enzyme digestion can benefit analyte recovery, ensure conversion of FMN and FAD, and aid in sample cleanup. For certain foods such as milk, dairy products, and eggs where the predominant form is free riboflavin or loosely bound forms (not FMN or FAD), acid and enzyme conversion is not always necessary for accurate riboflavin assay.[15] Russell and Vanderslice[62] reported that approximately 85% of the riboflavin in pasteurized whole milk was riboflavin with the remainder as FMN and FAD. No detectable coenzyme forms were present in raw and cooked whole egg or raw egg yolk. Various studies have used simple extraction procedures for the analysis of riboflavin in milk and dairy products.[35,122,107–110] Munoz et al.[107] deproteinized the sample with 10% lead acetate solution acidified to pH 3.2 with glacial acetic acid followed by filtration to prepare milk extracts for LC injection. For foods containing appreciable amounts of FMN and FAD, efficient conversion of the coenzymes to free riboflavin must be assured. Recommendations include the routine application of enzymatic hydrolysis by an enzyme preparation containing phosphatase activity to convert any remaining phosphorylated forms remaining after acid hydrolysis to free riboflavin.[15,59] Extraction procedures provided by the French Commission Générale d'Unification des Méthods d'Analyse[60] and the Standards, Measurement, and Testing Program (EUMAT)[58] provide excellent proven protocols for riboflavin extraction. The French study combined with the European Measurement and Testing program provide good insight into extractions useful for riboflavin, thiamin, and vitamin B_6. A more recent study by Ndaw et al.[61] provides a combined enzyme extraction with α-amylase, papain, and acid phosphatase that releases in a simple digestion step phosphorylated and protein-bound forms of riboflavin, thiamine, and vitamin B_6. This procedure was designed to be applicable to LC/fluorescent methods and is discussed fully in Chapter 14. Procedures are available for riboflavin assay of specific matrices such as wine,[123] flours,[124] and feeds.[125]

As discussed previously, riboflavin extraction from biologicals avoids hydrolysis if FMN and FAD are to be maintained intact. Capo-chichi et al.[112] extracted the flavins from plasma with TCA precipitation of the protein and solid-phase extraction with C_{18} Sep-Pack cartridges. This procedure is provided in Section 7.4.

7.3.2.3.2 Chromatography parameters

Supports and mobile phases

Various supports have been used for riboflavin chromatography. C_{18} stationary phases are most commonly used for total riboflavin analysis in foods and biologicals (Tables 7.7). Other supports such as C_{10}, $-NH_2$, hydrophilic gel, and normal-phase chromatography on silica have been less frequently, although successfully, applied. Mobile phases consist of mixtures of methanol or acetonitrile with water or buffers. Ion-pair chromatography with heptane or hexane sulfonate or triethyl- or tetrabutyl ammonium phosphate can improve resolution, but ion-pairs have not been needed in most published resolution systems.

Methods for the quantitation of riboflavin, FMN, and FAD use polymer-based columns (PLRP-5, Polymer Laboratories) of polystyrene/divinylbenzene resin to avoid stability problems noted for silica-based supports under the column conditions required for FMN and FAD stability. PLRP-5 was used by Russell and Vanderslice[62] with gradient elution by acetonitrile/0.1% sodium azide:10 mM citrate-phosphate buffer, pH 5.5 (3:97 to 6:94 to 14:86) to effectively resolve riboflavin, FMN, FAD, and 7-ethyl-8-methyl-riboflavin (internal standard) in various food extracts. Lopez-Anaya and Mayersohn[100] used a macroporous copolymer support (PRP-1, Hamilton) to isocratically resolve riboflavin, FMN, and FAD in plasma and urine with acetonitrile:water:10% trifluoroacetic acid:phosphoric acid (14:84:1.5:0.09), pH 1.8.

Table 7.7 shows a small but selective segment of the many successful chromatography systems available in the literature for riboflavin chromatography. The efficient coupling of riboflavin analysis and analysis of other water-soluble vitamins, primarily thiamine, into simultaneous assays requiring a single LC injection are discussed in Chapter 14.

7.3.2.3.3 Detection. Fluorescence detection (Ex $\lambda = 440\text{–}450$, Em $\lambda = 530$) is sensitive and specific for riboflavin quantitation after LC resolution. Detection limits for riboflavin by fluorescence is 1 pmol (0.38 ng) compared to 30 pmol (11 ng) by UV detection at 254 nm.[11] Ultraviolet detection is adequate for pharmaceuticals and enriched foods. However, its use for samples with lower, naturally occurring vitamin levels most likely will require concentration and cleanup of the extract before chromatography. Vidal-Valverde and Reche[111] treated acid and enzyme hydrolysates of foods by Florisil and C_{18} Sep-Pak chromatography to allow use of UV detection at 254 nm.

Conversion of riboflavin to lumiflavin was effectively used to increase the sensitivity of fluorescence detection of riboflavin at low levels in meat and meat products (Section 7.4).[22] Riboflavin in extracts prepared by autoclaving in 0.1 N HCl, digestion by papain and takadiastase, and TCA precipitation of protein was converted to lumiflavin by UV irradiation at pH 10–12. The lumiflavin was extracted with chloroform. The chloroform extract was injected into the LC system with fluorescence detection at Ex $\lambda = 270$, Em $\lambda = 418$. The detection limit was 0.02 ng per injection.

At present, few analytical procedures have applied mass spectrometry to the analysis of riboflavin. This is most likely due to the good capability of fluorescence to provide necessary sensitivity and specificity to the LC analysis of foods and biologicals. Hardwick et al.[120] applied LC with fluorescence detection and tandem MS/MS to the analysis of riboflavin and photodegradation products, including lumichrome, in blood products (Figure 7.7). Characteristics of riboflavin determined in this study are provided in Table 7.8.

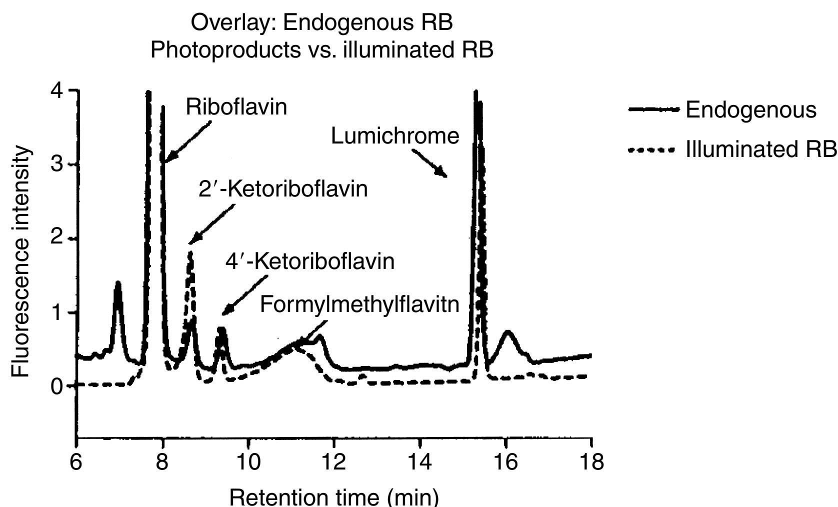

Figure 7.7 Chromatogram of a platelet concentrate containing photodegradation products of riboflavin. (Reproduced from Hardwick, C. C., Herivel, T. R., Hernandez, S. C., Ruane, P. H., and Goodrich, R. P., *Photochem. Photobiol.*, 80, 609, 2004. With permission.)

Table 7.8 Properties of Riboflavin and Some Photodegradation Products

Compound	UV_{max} (nm)	MS/MS transitions (amu)
Riboflavin	223, 267, 374, 444	375.1 > 255.1
Lumichrome	218, 260, 352	240.9 > 198.1
2′-Keto-flavin	224, 268, 372	373.1 > 241.3
	446	373.1 > 255.1
4′-Keto-flavin	222, 268, 374	373.1 > 241.3
	446	373.1 > 255.1
Formylmethyl flavin	222, 268, 372, 446	282.9 > 240.2

Source: Reproduced from Harwick, C.C., Hervel, T.R., Hernandez, S.C., Ruane, P.M., and Goodrich, R.P., *Photochem. Photobiol.*, 80, 609, 2004. With permission.

Advantages of sensitivity and selectivity make fluorescence detection of the flavins a clear choice over UV detection. Increased use of mass spectrometry will add increased detection capabilities in the coming years.

Internal Standards

Internal standards for riboflavin and flavin coenzyme analysis are not generally available. Compounds used in past research include 2,2,9-diphenic acid, nicotinamide, p-hydroxybenzoic acid, acetosalicylic acid, theobromine, isoriboflavin, sorboflavin, and 7-ethyl-8-methyl riboflavin. All of these compounds have different structural properties compared to riboflavin except the riboflavin analogs isoriboflavin (8-demethyl-6-methyl riboflavin),[114] sorboflavin (glucityl side chain on the ribityl chain),[102] and 7-ethyl-8-methyl-riboflavin.[62] Bilic and Sieber[102] provide detailed synthesis instructions for sorboflavin. Bötticher and Bötticher[126] and, more recently, Capo-chichi et al.[112] used galactoglavin as an internal standard for flavin assay of plasma.

7.4 Method protocols

Liquid Chromatographic Analysis of Riboflavin Vitamers in Foods Using Fluorescence Detection

J. Agric. Food Chem., 52, 1789, 2004[106]

Principle

Riboflavin, FMN, and FAD are extracted with acetonitrile and phosphate buffer. Flavins are chromatographed on C^{16} amide stationary phase with fluorescence detection.

Chemicals

- KH_2PO_4
- KOH
- H_3PO_4
- FMN
- FAD
- Riboflavin
- Acetonitrile

Apparatus

- HPLC
- Fluorescence detector, multichannel
- Variable power ultrasonic bath
- Vibratory stirrer
- Centrifuge

Sample Extraction

- Weigh 1–2 g sample into extraction tube.
- Add 10 mL acetonitrile and homogenize with the vibratory stirrer, 10 min.
- Add 10 mL of 10 mM phosphate buffer, pH 5.0.
- Centrifuge at 6000 rpm, 5 min.
- Dilute supernatant to 25 mL with the phosphate buffer.
- Filter through 0.45 µm filter, inject.

Chromatography

Column	15 cm × 4.6 mm
Stationary phase	Discovery RP-Amide C_{16}, 5 µm (end-capped with TMS)
Mobile phase	MeCN:10 mM KH_2PO_4 (10:90), pH 5.0
Flow rate	1 mL min^{-1}
Column temperature	Ambient
Detection	Fluorescence, Ex $\lambda = 270$, Em $\lambda = 516$
Calculation	Standard addition, peak area

Analysis of Riboflavin and Riboflavin Cofactor Levels in Plasma by High-Performance Liquid Chromatography

J. Chromatogr. B, 739, 219, 2000[112]

Principle

Riboflavin, FMN, and FAD are extracted from plasma by solid-phase extraction on C_{18} Sep-Pack cartridges after protein precipitation with TCA. Flavins were chromatographed using reversed-phase chromatography on C_{18} with fluorescence detection.

Chemicals

- Trichloroacetic acid (TCA)
- Methanol
- Magnesium acetate
- Orthophosphoric acid
- KH_2PO_4
- Acetonitrile
- Riboflavin
- FMN
- FAD
- Galactoflavin

Apparatus

- HPLC
- Fluorescent detector

Plasma Extraction

- To 1 mL plasma, add galactoflavin (IS) to final concentration of 206 nM.
- Add 1 mL 15 mM magnesium acetate, incubate at 65°C, 15 min.
- Add 0.5 mL TCA.
- Centrifuge, 3200 g, 15 min, 4°C.
- Rinse pellet with 1 mL magnesium acetate, combine supernatants.
- Add supernatants onto a C_{18} Sep-Pack cartridge (conditioned) with 2 mL methanol and 2 mL of a 10 mM KH_2PO_4 and 15 mM magnesium acetate solution, pH 3.4.
- Elute flavin with 2 mL of the KH_2PO_4–magnesium acetate solution.

Chromatography

Column	25 cm × 4 mm
Stationary phase	C_{18}
Mobile phase	15% MeCN in the 10 mM KH2PO4-15 mM magnesium acetate solution
Flow rate	1 mL min^{-1}
Column temperature	Ambient
Detection	Fluorescence, Ex $\lambda = 445$, Em $\lambda = 530$
Calculation	Internal standard, galactoflavin

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chapter eight

Niacin

8.1 Review

Niacin deficiency "Pellagra" was described by Casel in Spain in 1735 and by Pugati in Italy in 1755. Pellagra is derived from the Italian term "pelle agra" for rough skin. Recognition of the disease as a dietary deficiency did not occur until Goldberger showed that pellagra was not an infectious disease and could be induced by the lack of a nutritional factor in corn. The factor was called the "pellagra preventive factor."[1,2] Goldberger's studies led to the recognition of black tongue disease in dogs that were fed diets that caused pellagra in humans. Consequently, in 1937, nicotinic acid was recognized as a curative factor for black tongue in dogs[3] and for pellagra.[4]

Although corn consumption as a dietary staple was linked to pellagra incidence for centuries, the etiology was not clearly defined until the relationship between dietary tryptophan and its conversion to niacin was understood. Krehl et al.[5] showed that tryptophan could replace nicotinic acid and prevent pellagra-like symptoms in rats. Niacin deficient rats fed corn diets were cured by feeding casein, a good tryptophan source. Later work showed that tryptophan is a precursor for the biosynthesis of nicotinic acid.[6] Studies conducted from 1961 to 1980 designed to determine the contribution of dietary tryptophan to the niacin requirement led to the accepted interconversion factor of 60 to 1.[7] Sixty mg of dietary tryptophan is considered equivalent to 1 mg of niacin. The concept of niacin equivalents (NEs) was introduced by Horwitt et al.[8] in 1981 (1 NE equals 1 mg of niacin and 60 mg of dietary tryptophan). Dietary Reference Intakes (DRIs) are reported as mg NE to include the impact of metabolic conversion of tryptophan to the human niacin requirements.[7] The historically significant role of corn diets in pellagra incidence is explained by the low levels and poor bioavailability of both tryptophan and nicotinic acid in corn.

Marginal niacin deficiency shows multiple symptoms including insomnia, loss of appetite, weight and strength loss, soreness of the tongue and mouth, indigestion, abdominal pain, burning sensation in various parts of the body, vertigo, headache, numbness, nervousness, distractibility, apprehension, mental confusion, and forgetfulness.[9] Pellagra or frank niacin deficiency includes symptoms of dermatosis, dementia, and diarrhea (Three-D Disease). When death occurs, the phrase Four-D Disease applies. In developed countries, alcoholics are at risk of developing pellagra. Pellagra continues to be problematic in dietary deficient areas where corn and other cereals are major dietary staples. Clinical status tests include the measurement of the niacin metabolites *N*-methylnicotinamide (NMN) and *N*-methyl-2-pyridone-5-carboxamide (2-pyridone), *N*-methyl-4-pyridone-3-carboxamide (4-pyridone) and *N*-methyl-6-pyridone-3-carboxamide (6-pyridone) in the urine.[10] Blood levels of niacin or its cofactors and metabolites are not used as status indicators owing to

inconsistent results or unsatisfactory methodology. Levels in plasma respond rapidly to dietary intake and do not provide an accurate index of tissue stores.[10] Analysis of urinary excretion of NMN and one or more of the pyridone metabolites by liquid chromatography (LC) is used as a measure of niacin status. In adults, at niacin intakes ≤6 NE d^{-1}, urinary excretion of NMN and 2-pyridone falls to less than 8.8 μmol d^{-1}.[10]

In the United States, calculated mg NE intakes are 27 for women and 41 for men.[11] The Reference Daily Intake (RDI) established by the Food and Drug Administration for nutritional labeling is 20 mg NE.[12] Recommended Dietary Allowances (RDAs) range from 16 mg d^{-1} for adult males to 18 mg d^{-1} for females during pregnancy (Table 8.1).[7] Therefore, intakes of mg NE by most individuals in the United States exceeds RDI and RDA amounts.

Owing to the contribution of dietary tryptophan to mg NE intake, foods containing balanced protein are important dietary mg NE sources. Table 8.2 provides a ranking of important niacin sources in the U.S. diet derived from the United States Department of Agriculture (USDA) Nutrient Database (SR 19).[13] These data are based upon niacin content (mg 100 g^{-1}) and not NE levels. High protein foods containing tryptophan can contribute to NE intake levels even if niacin is quite low in the food. As an example, whole milk (3.25% butter fat) contains only 0.1 mg 100 g^{-1} of niacin. Tryptophan at 75 mg 100 g^{-1} contributes an additional 1.25 mg NE 100 g^{-1}.

Few foods, unless fortified, contain free niacin in appreciable quantities. In unprocessed foods, naturally occurring niacin is present mostly in the pyridine coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). Nicotinic acid and nicotinamide serve as precursors for the synthesis of NAD and NADP. The dietary forms (NAD and NADP) are hydrolyzed in the intestinal mucosa, and nicotinic acid is converted to nicotinamide during coenzyme biosynthesis. The metabolically active form in the coenzymes is nicotinamide, the primary circulating form of niacin.[14] NAD and NADP function in oxidation–reduction reactions. Nicotinamide acts as an electron acceptor or hydrogen donor. Jacob and Swendseid[14] summarized the function of NAD as an electron carrier for intracellular respiration and a codehydrogenase involved in the oxidation of fuel molecules such as glyceraldehyde 3-phosphate, pyruvate, and lactate. NADP functions as a hydrogen donor in reductive processes for biosynthesis of fatty acids and steroids, and as a codehydrogenase for oxidation of glucose 6-phosphate to ribose 5-phosphate in the pentose phosphate pathway.[14] Much recent interest has centered on the role of nicotinic acid adenine dinucleotide phosphate (NAADP) and its function as an intracellular second messenger.[15] It is a powerful stimulator of intracellular Ca^{+2} release. NAD and NADP enzyme dependent reactions are summarized in Table 8.3.[16] Ball[17] provides an excellent review of niacin absorption, transport, and metabolism.

A well-recognized therapeutic role of niacin is its ability to lower serum cholesterol.[18–27] Ganji et al.[19] summarized the effects of niacin therapy on lipid and lipoprotein metabolism as a beneficial reduction in triglyceride and apolipoprotein-B containing lipoproteins. This occurs primarily through

1. Inhibition of fatty acid mobilization from adipose tissue, resulting in decreased availability of fatty acids for triglyceride synthesis.
2. Inhibition of fatty acid synthesis in hepatocytes and the esterification of diacylglycerol to triglycerides, resulting in decreased triglyceride synthesis.
3. Inhibition of triglyceride synthesis decreases lipidation of apo B and increases intracellular apo B degradation, which decreases concentration of very low density lipoprotein and low density lipoprotein (LDL).
4. Inhibition of the high density lipoprotein (HDL) catabolism receptor that decreases HDL catabolism, with increased HDL catabolism resulting in increased HDL half-life and cholesterol efflux and reversed cholesterol transport.

Table 8.1 Dietary Reference Intakes and Tolerable Upper Intake Levels for Niacin Equivalents

Life stage	DRI (mg d^{-1})	UL (mg d^{-1})
Infants (months)		
0–6	2	[a]
7–12	4	[a]
Children (years)		
1–3	**6**	10
4–8	**8**	15
Males (years)		
9–13	**12**	20
14–18	**16**	30
19–30	**16**	35
31–50	**16**	35
51–70	**16**	35
>70	**16**	35
Females (years)		
9–13	**12**	20
14–18	**14**	30
19–30	**14**	35
31–50	**14**	35
51–70	**14**	35
>70	**14**	35
Pregnancy (years)		
≤18	**18**	30
19–30	**18**	35
31–50	**18**	35
Lactation (years)		
≤18	**17**	30
19–30	**17**	35
31–50	**17**	35

Bold type: Recommended Dietary Allowance; ordinary type: Adequate Intake (AI).

[a] Not possible to establish; source of intake should be formula and food only.

Source: Food and Nutrition Board, Institute of Medicine, *Dietary Intakes for Thiamin, Riboflavin, Niacin, Vitamin B_6, Folate, Vitamin B_{12}, Pantothenic Acid, Biotin, and Choline*, National Academy of Sciences Press, Washington, DC, 2000, pp. 123–149.

Many additional roles have been identified for niacin and its metabolites. Biological functions in addition to action as a cofactor in oxidation-reduction reactions at all levels of metabolism include transcriptional regulation and interactions at the gene level generally affecting longevity and the quality of life.[28–32]

8.2 Properties

8.2.1 Chemistry

Niacin refers to nicotinic acid ($C_6H_5O_2N$) and nicotinamide ($C_6H_6ON_2$), which have equal biological activity. Structures are given in Figure 8.1. Nicotinic acid is, chemically, pyridine 3-carboxylic acid and nicotinamide is pyridine 3-carboxylic acid amide. The acid and amide

Table 8.2 Niacin Content of Various Foods

Description	NDB no.	mg 100 g^{-1}	Description	NDB no.	mg 100 g^{-1}
Cereals ready-to-eat, GENERAL MILLS, Whole Grain TOTAL	08077	88.1	Lamb, domestic, loin, separable lean and fat, trimmed for 1/4″ fat, choice, cooked, broiled	17024	7.1
Cereals ready-to-eat, KELLOGG, KELLOGG'S Complete Wheat Bran Flakes	08028	69.0	Chicken, broilers or fryers, dark meat, meat only, cooked, fried	05044	7.1
Cereals ready-to-eat, KELLOGG, KELLOGG'S PRODUCT 19	08058	66.7	Wheat flour, white, cake, enriched	20084	6.8
Cereals ready-to-eat, GENERAL MILLS, TOTAL Corn Flakes	08246	66.7	Turkey, all classes, light meat, cooked, roasted	05186	6.8
Malted drink mix, natural, with added nutrients, powder	14309	48.6	Fish, tuna salad	15128	6.7
Cereals ready-to-eat, GENERAL MILLS, TOTAL Raisin Bran	08247	36.4	Fish, salmon, sockeye, cooked, dry heat	15086	6.7
Cereals ready-to-eat, GENERAL MILLS, FROSTED WHEATIES	08266	33.3	Wheat flour, whole-grain	20080	6.4
Cereals ready-to-eat, GENERAL MILLS, WHEATIES	08089	33.3	Buckwheat flour, whole-groat	20011	6.2
Cereals ready-to-eat, KELLOGG, KELLOGG'S FROOT LOOPS	08030	24.2	Wheat flour, white, all-purpose, enriched, bleached	20081	5.9
Cereal ready-to-eat, KELLOGG, KELLOGG'S SPECIAL K	08067	23.0	Wheat flour, white, all-purpose, self-rising, enriched	20082	5.8
Cereal ready-to-eat, KELLOGG, KELLOGG'S RICE KRISPIES	08065	21.5	Pork, fresh, loin, broiled	10042	5.5
Cereals ready-to-eat, QUAKER, CAP'N CRUNCH	08010	21.2	Bulgur, dry	20012	5.1
Cereals ready-to-eat, GENERAL MILLS, CHEERIOS	08013	19.2	Cornmeal, degermed, enriched, yellow	20022	5.0
Peanuts, oil roasted, with salt	16089	13.8	Barley, pearled, raw	20005	4.6
Chicken, broilers or fryers, breast, meat only, cooked, roasted	05064	13.7	Cornmeal, self-rising, degermed, enriched, yellow	20025	4.6
Fish, tuna, light, canned in water, drained solids	15121	13.3	Mushrooms, cooked, boiled, drained, without salt	11261	4.5
Fish, tuna, light canned in oil, drained solids	15119	12.4	Rice, white, long-grain, regular, raw, enriched	20044	4.2
Chicken, broilers or fryers, breast, meat and skin, cooked, fried, batter	05058	10.5	Fast foods, cheeseburger, regular, double patty, plain	21092	3.9
Fish, trout, rainbow, farmed, cooked, dry heat	15241	8.8	Rice, white, long-grain, parboiled, enriched, dry	20046	3.6
Wheat flour, white, bread, enriched	20083	7.6	Couscous, dry	20028	3.5
Beef, top sirloin, separable lean and fat, trimmed to 1/8″ fat, all grades, cooked, broiled	13930	7.2	Tomato products, canned, paste, without salt added	11546	3.1

Source: Data from United States Department of Agriculture, Agricultural Research Service, 2006, USDA Nutrient Database for Standard Reference, Release 19, Nutrient Data Laboratory Home Page, http://www.nal.usda.gov/fnic/foodcomp, Riverdale, MD, Nutrient Data Laboratory, USDA.

Table 8.3 NAD and NADP Dependent Enzymes

Enzyme	Pyridine nucleotide
Carbohydrate metabolism	
3-Phosphoglyceraldehyde dehydrogenase	NAD(H)
Glucose-6-phosphate dehydrogenase	NADP(H)
6-Phosphogluconate dehydrogenase	NADP(H)
Lactate dehydrogenase	NAD(H)
Alcohol dehydrogenase	NAD(H)
Lipid metabolism	
α-Glycerophosphate dehydrogenase	NAD(H)
β-hydroacyl CoA dehydrogenase	NAD(H)
3-Ketoacyl ACP reductase	NADP(H)
Enoyl-ACP-reductase	NADP(H)
3-Hydroxy-3-methylglutaryl-CoA reductase	NADP(H)
Amino acid metabolism	
Glutamate dehydrogenase	NAD(H)/NADP(H)
Other	
Glutathione reductase	NADP(H)
Dihydrofolate reductase	NADP(H)
Thioredoxin-NADP reductase	NADP(H)
4-Hydroxybenzoate hydroxylase	NADP(H)
NADH dehydrogenase/NADH-ubiquinone reductase complex	NAD(H)
NADPH-cytochrome P450 reductase	NADP(H)

Source: From Combs, G.F., Jr., *The Vitamins, Fundamental Aspects in Nutrition and Health*, Academic Press, New York, 1992, Chap. 12.

forms are readily interconvertible and nicotinic acid is converted to the amide in formation of NAD and NADP. Structures of the coenzymes are given in Figure 8.2.

8.2.1.1 General properties

Physical properties of nicotinic acid, nicotinamide, and the coenzymes are given in Table 8.4. Nicotinic acid is the USP reference standard. Both compounds are white needle-shaped crystals. Nicotinamide is more water-soluble (100 g 100 mL^{-1}) than nicotinic acid (1.67 g 100 mL^{-1}).[33–36] The compounds are odor free. Nicotinamide exhibits a bitter flavor, which can be detected at higher levels in fortified foods. Both free niacin forms are bases and form quaternary ammonium salts when dissolved in acid solutions.[34] Nicotinic acid is amphoteric and forms carboxylic acid salts in basic solution.[34] Both compounds are available for fortification and supplementation. Nicotinamide is used in dry and liquid products, and nicotinic acid is used in products in which its lower solubility does not pose problems.[34] Bitterness of nicotinamide can be masked by enrobing techniques. Analysts or production workers must handle nicotinic acid carefully. It is a powerful vasodilator, leading to a rapid flushing through inhalation of the dust. Oral dosages designed to lower serum cholesterol are great enough to cause the flushing response in such patients.[35]

8.2.1.2 Spectral properties

Nicotinic acid and nicotinamide show similar absorption properties with absorption maxima near 260 nm. Absorption intensity is pH dependent. The ultraviolet (UV) absorption spectrum of niacinamide standard and chromatographically resolved niacinamide are shownin Figures 8.3 and 8.4. The free forms of the vitamin do not fluoresce. Coenzymes

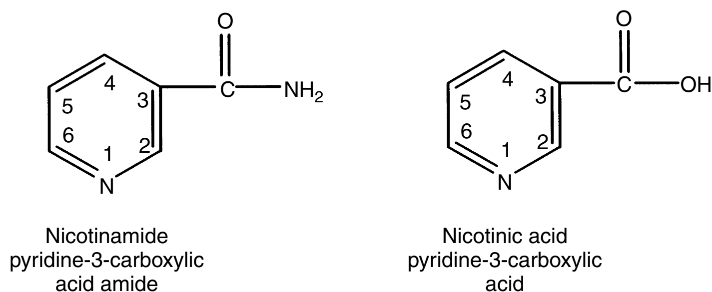

Figure 8.1 Structures of nicotinic acid and nicotinamide.

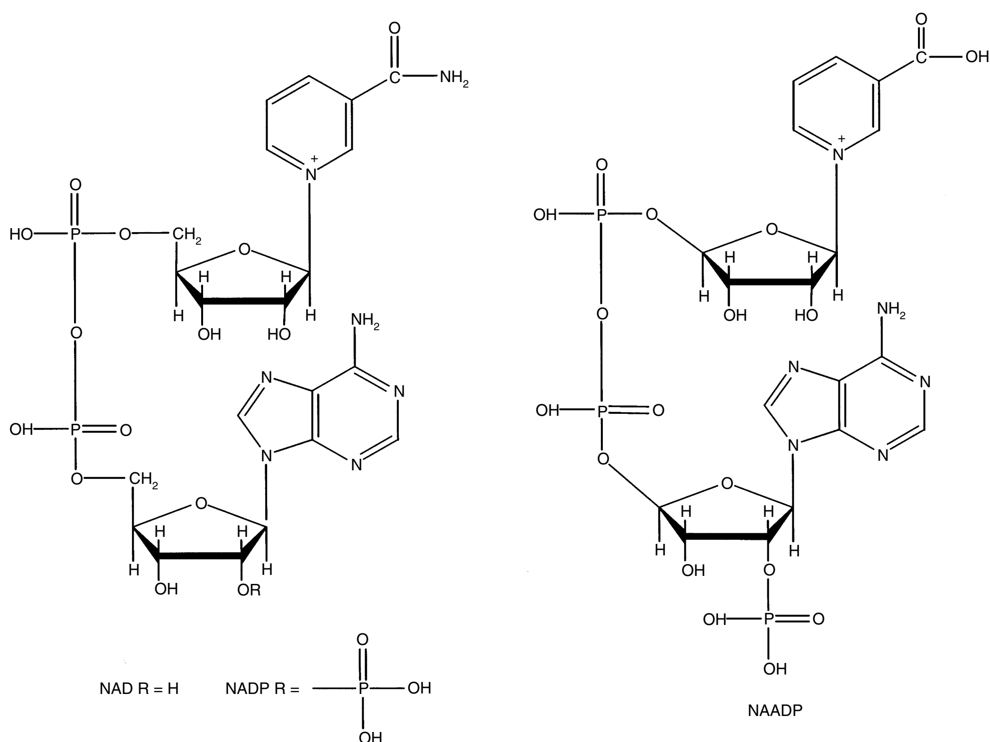

Figure 8.2 Structures of nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), and nicotinic acid adenine dinucleotide phosphate (NAADP).

show two UV maxima at 260 nm (nicotinamide ring) and 340 nm (adenine ring).[2] The coenzymes fluoresce at 470 nm when excited at 260 nm or 340 nm.[2] The lack of fluorescence of nicotinic acid and nicotinamide and relatively low specificity of UV detection is problematic for LC analysis of niacin in biological materials. When UV detection is used, most extraction procedures require time-consuming cleanup steps to produce interference free chromatograms (Section 8.3.3).

8.2.2 Stability

Niacin is the most stable water-soluble vitamin.[37] Biological activity is not affected by thermal processing, light, acid, alkali, or oxidation. Owing to its stability, acid or alkali hydrolysis can be used for extraction from biological samples. Such conditions free the niacin from coenzyme structures and destroy the sample matrix. From a food processing

Table 8.4 Physical Properties of Nicotinic Acid and Nicotinamide

Substance[a]	Molar mass	Formula	Solubility	Melting point °C	Crystal form	λ_{max}(nm)	Absorbance[b]		
							$E^{1\%}_{1cm}$	$\varepsilon \times 10^{-3}$	Solvent
Nicotinic acid CAS No. 58–67-6 **6612**	123.11	$C_6H_5NO_2$	Water 1.67 g 100 mL^{-1} Ethyl alcohol 0.73 g 100 mL^{-1} Soluble in alkali hydroxides and carbonates, propylene glycol Insoluble in ether	236.6	Needles	260	[227]	2.8	50 mM potassium phosphate buffer, pH 7.0
Nicotinamide CAS No. 68–92–0 **6574**	122.13	$C_6H_6N_2O$	Water 100 g 100 mL^{-1} Ethyl alcohol 66.6 g 100 mL^{-1} Soluble in glycerol	128–131	Needles	261	478	[5.8]	0.1 N H_2SO_4

[a] Common or generic name; CAS No.: Chemical Abstract Service number, bold print designates the Merck Index monograph number.

[b] Values in brackets are calculated from corresponding ε or $E^{1\%}_{1\,cm}$ values.

Sources: From Eitenmiller, R. R. and DeSouza, S., Niacin, 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, Chap. 15; Ball, G. F. M., Chemical and biological nature of the water-soluble vitamins, In *Water-Soluble Vitamin Assays in Human Nutrition*, Chapman & Hall, New York, 1994, Chap. 2; Shibata, K. and Taguchi, H., Nicotinic acid and nicotinamide In *Modern Chromatographic Analysis of Vitamins*, 3rd ed., De Leenheer, A. P., Lambert, W. E., and Van Bocxlaer, J., Eds., Marcel Dekker, New York, 2000, Chap. 7; Budavari, S., *The Merck Index*, 13th ed., Merck and Company, Whitehouse Station, NJ, 2001, pp. 1168–1170.

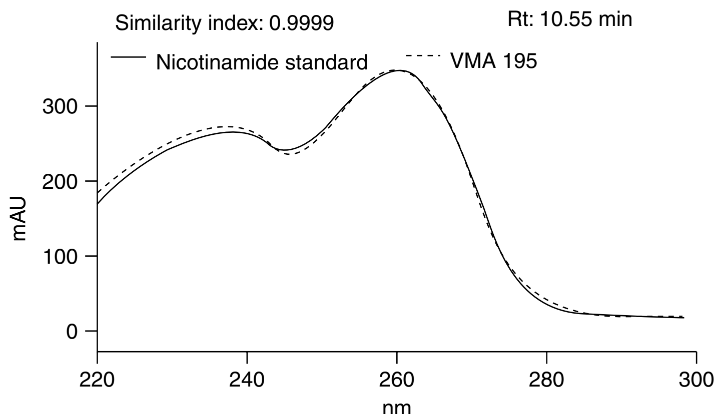

Figure 8.3 UV absorption spectra of nicotinamide standard and nicotinamide resolved from VMA-195, AACC cereal reference. (Reproduced from LaCroix, D. E., Wolf, W. R., and Kwansa, A. L., *Cereal Chem.*, 82, 277, 2005. With permission.)

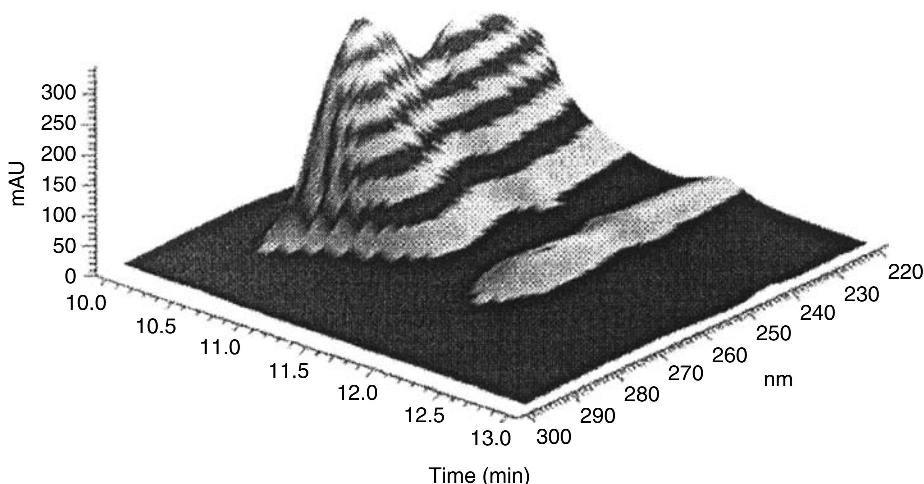

Figure 8.4 3-D view nicotinamide peak resolved from VMA-195. (Reproduced from LaCroix, D. E., Wolf, W. R., and Kwansa, A. L., *Cereal Chem.*, 82, 277, 2005. With permission.)

standpoint, processing and cooking procedures do not inactivate niacin. Leaching is usually the primary route for loss during food preparation.[16,37] Dairy processing operations do not affect niacin content.[34] Leaching of available niacin from foods is recognized as the primary route of loss during processing and cooking. Prodanov et al. [38] reported that as much as 46% of available niacin was lost from lentils through soaking and cooking (Figure 8.5).

8.2.3 Bioavailability

Bioavailability of niacin in unfortified foods is often low. Significant proportions of total niacin can exist in bound forms that are not absorbed by the human or cannot be utilized if absorbed. Without specific processing, such as alkali treatment of corn, the bound vitamin

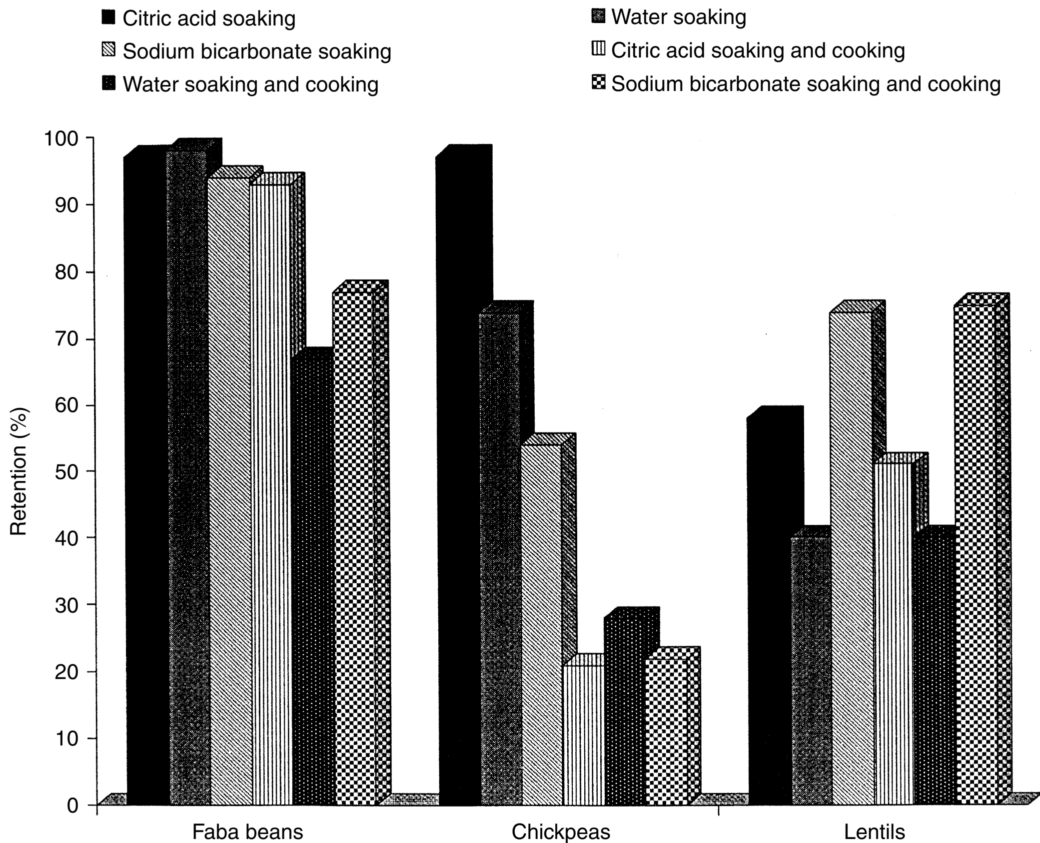

Figure 8.5 Effect of processing on available niacin in legumes. (Reproduced from Prodanov, M., Sierra, I., and Vidal-Valverde, C., *Food Chem.*, 84, 271, 2004. With permission.)

is partially unavailable. As much as 50% of the total niacin in nonenriched wheat and wheat products is unavailable.[39] Other cereals are assumed to contain significant amounts of bound "unavailable" niacin. Koetz et al.[40] identified 3-*O*-nicotinyl-*O*-glucose as an acid hydrolysis product of wheat bran; thus, establishing that the ester linkage between nicotinic acid and the 3-position of glucose as one mechanism for formation of unavailable niacin in the plant kingdom. Unavailable forms of niacin in foods have never been fully characterized. Other complex forms include amide linkages between amino groups of proteins and peptide and the carboxyl group of nicotinic acid. Protein-bound nicotinic acid and glucose esters are thought to be partially available to humans.[41,42] The methylated derivative of nicotinic acid, l-methylnicotinic acid, or trigonelline, is not biologically active. However, it is converted to nicotinic acid by thermal processing.[16] Treatment of corn with alkali liberates bound niacin and improves the bioavailability to humans.[43] Alkali processing of tortilla flour largely prevents pellagra in Central and South America where corn is the primary staple cereal.[37]

It is of significance to accurate assessment of niacin intake that extraction methods that use acid hydrolysis do not hydrolyze most bound forms found in cereals. Such methods do not overestimate available niacin. Extraction by alkaline hydrolysis will liberate nicotinic acid from the macromolecules. Alkali hydrolysis, therefore, measures total niacin, but overestimates the amount naturally available from the food.[44]

8.3 Methods

Several time-tested methods based on chemical and microbiological assays are still routinely used. High performance liquid chromatography (HPLC) methods are available; however, LC methods rely on UV detection, and sensitivity and selectivity remains a problem. Some gas chromatography (GC) procedures are available, but these methods have seen little use. Regulatory methods often rely on chemical procedures based on the classical König reaction or microbiological assay by *Lactobacillus plantarum* ATCC No. 8014. The following section discusses chemical, microbiological, HPLC methods as well as newer methods based on capillary electrophoresis and mass spectrometry coupled to liquid or gas chromatographs. Methodology reviews include Eitenmiller and DeSouza,[33] Ball,[34] Shibata and Taguchi,[35] Eitenmiller and Landen,[37] and Lumley.[44] Various handbook and compendium methods are summarized in Table 8.5.[45–57]

8.3.1 Chemical

The colorimetric determination of niacin is based upon the König reaction in which nicotinic acid and nicotinamide react with cyanogen bromide, yielding a pyridinium compound (Figure 8.6). Rearrangement produces derivatives that couple with aromatic amines to form colored compounds in the polymethine dye family with absorption maxima at 436 nm.[33,58] Under properly controlled conditions, color produced is proportional to the niacin content. Factors that must be controlled include reaction temperature, pH, the choice of an aromatic amine for maximum color development, and the preparation of a proper blank for background correction.[59] Recent work has coupled the König reaction with reversed-phase HPLC for assay of nicotinic acid and nicotinamide.[60] Other modifications allow analysis of small quantities of nicotinic acid in the presence of nicotinamide.[61]

Current Association of Official Analytical Chemists (AOAC) International[47] colorimetric methods use the König reaction. The methodology was originally developed by Pelletier and Campbell.[62]

AOAC Official Method 961.14, Niacin and Niacinamide in Drugs, Foods, and Feeds, Colorimetric Method AOAC Official Methods of Analysis of *AOAC Int*ernational, 45.1.10.

Method 961.14 is the manual *AOAC Int*ernational Method. Procedural steps include the following as summarized by Eitenmiller and DeSouza.[33]

8.3.1.1 Sample preparation

1. *Pharmaceuticals*: Prepare a sample containing at least five tablets or capsules by dispersing in small volume of water with heating. Tablets may be ground. Cool and transfer to volumetric flask and dilute to volume so that final solution contains 50 to 200 mg niacin per mL. Pipette 10 mL aliquot into 250 mL Erlenmeyer flask and add 10 mL concentrated HCl. Evaporate on hot plate to approximately 2 mL, cool, and add 25–50 mL of water. Adjust pH to 2.5–4.5 with 40% NaOH or KOH. Adjust volume with water so that final volume contains approximately 4 mg niacin per mL. If cloudy, filter or centrifuge.
2. *Noncereal foods and feeds*: Weigh approximately one ounce (25–30 g) of sample into 1000 mL Erlenmeyer flask and disperse with 200 mL of 1 N H_2SO_4. Autoclave for 30 min at 15 psi, cool, and adjust pH to 4.5 with 10 N NaOH. Dilute to 250 mL with water and filter. Weigh 17 g of $(NH_4)_2SO_4$ into 50 mL volumetric flask and pipette in 40 mL of sample solution. Dilute to volume with water and shake vigorously. Clarify by filtration or centrifugation, and use 1 mL for niacin quantitation.

Table 8.5 Regulatory and Handbook Methods of Analysis for Niacin, Nicotinic Acid, and Nicotinamide

Source	Form	Methods and application	Approach	Most current cross-reference
***U.S. Pharmacopeia National Formulary*, 2006, USP 29/NF 24, Dietary Supplements Official Monographs**[45]				
1. Pages 2392–2394, 2396, 2399, 2419–2421	Nicotinic acid Nicotinamide	Niacin/niacinamide in oil- and water-soluble vitamin capsules/tablets w/wo minerals	Method 1—LC 280 nm Method 2—LC 254 nm Method 3—LC 270 nm	None
2. Pages 2428–2430, 2432, 2436	Nicotinic acid Nicotinamide	Niacin/niacinamide in water-soluble vitamin capsules/tablets w/wo minerals	Method 1—LC 280 nm Method 2—LC 254 nm Method 3—LC 270 nm	None
3. Page 1520	Nicotinic acid	Niacin (NLT 99.0%, NMT 101.0%)	Spectrophotometric 262 nm	None
4. Page 1521	Nicotinic acid	Niacin in tablets	LC 262 nm	None
5. Pages 1520–1521	Nicotinic acid	Niacin in injection	Colorimetric 450 nm	None
6. Page 1522	Nicotinamide	Niacinamide in injection/tablets	Colorimetric 450 nm	None
7. Page 1521–1522	Nicotinamide	Niacinamide (NLT 98.5%, NMT 101.5%)	LC 254 nm	None
***British Pharmacopoeia*, 2007**[46]				
1. Page 1463	Nicotinamide	Nicotinamide	Titration	None
2. Pages 1466–1467	Nicotinic acid	Nicotinic acid	Titration	None
3. Pages 2784–2785	Nicotinamide	Nicotinamide tablets	Spectrophotometric 262 nm	None
4. Page 2785	Nicotinic acid	Nicotinic acid tablets	Titration	None
5. Pages 2989–2990	Nicotinamide	Vitamins B and C injection	LC 280 nm	None

Continued

Table 8.5 (Continued)

Source	Form	Methods and application	Approach	Most current cross-reference
***AOAC Official Methods of Analysis*, 18th ed., 2005**[47]				
1. 45.2.04	Nicotinic acid Nicotinamide	AOAC Official Method 944.13, Niacin and Niacinamide in Vitamin Preparations	Microbiological	*J. Assoc. Off. Anal Chem.*, 42, 529, 1959[48]
2. 45.1.10	Nicotinic acid Nicotinamide	AOAC Official Method 961.14, Niacin and Niacinamide in Drugs, Food and Feeds	Colorimetric 470 nm	*J. Assoc. Off. Anal. Chem.*, 58, 799, 1975[49]
3. 45.1.11	Nicotinic acid Nicotinamide	AOAC Official Method 975.41, Niacin and Niacinamide in Cereal Products	Automated Colorimetric 470 nm	*J. Assoc. Off. Anal. Chem.*, 58, 799, 1975[49]
4. 45.1.12	Nicotinic acid Nicotinamide	AOAC Official Method 981.16, Niacin and Niacinamide in Foods, Drugs and Feed	Automated Colorimetric 470 nm	*J. Assoc. Off. Anal. Chem.*, 62, 1027, 1979[50]
5. 45.1.13	Nicotinamide	AOAC Official Method 968.32, Niacinamide in Multivitamin Preparations	Spectrophotometric 550 nm	*J. Assoc. Off. Anal. Chem.*, 51, 828, 1968[51]
6. 50.1.19	Nicotinic acid Nicotinamide	AOAC Official Method 985.34, Niacin and Niacinamide in Ready-To-Feed Milk-Based Infant Formula	Microbiological	*J. Assoc. Off. Anal. Chem.*, 68, 514, 1985[52]

American Association of *Cereal Chemists*, Approved Methods, 1996, vol. 2[53]				
1. AACC 86–49	Nicotinic acid	Niacin in enriched concentrates	Colorimetric	
2. AACC 86–50	Nicotinic acid Nicotinamide	Niacin and nicotinamide in cereal products	Colorimetric 470 nm	*AOAC Int.*, 18th ed., 45.1.10, 2005;[47]
	Nicotinamide		400 nm	*J. Assoc. Off. Anal. Chem.*, 45, 449, 1962[54]
3. AACC 86–51	Nicotinic acid Nicotinamde	Niacin in cereal products	Microbiological	*AOAC Int.*, 18th ed., 45.2.04, 2005;[47] *Cereal Chem.*, 19, 553, 1982[55]
4. AACC 86–52	Nicotinic acid Nicotinamide	Niacin and niacinamide automated determination in cereal products	Automated Colorimetric 470 nm	*J. Agric. Food Chem.*, 22, 232, 1974;[56] *AOAC Int.*, 18th ed., 45.1.12, 2005[47]
Food Chemicals Codex, 5th ed., 2004[57]				
1. page 264	Nicotinic acid	Niacin (NLT 99.5%, NMT 101.0%)	Titration, NaOH	None
2. pages 264–265	Nicotinamide	Niacinamide (NLT 98.5%, NMT 101.0%)	Titration, perchloric acid	None
3. page 265	Niacinamide ascorbate	Niacinamide ascorbate (NLT 73.5% Ascorbic Acid) (NLT 24.5% Niacinamide)	Titration, perchloric acid	None

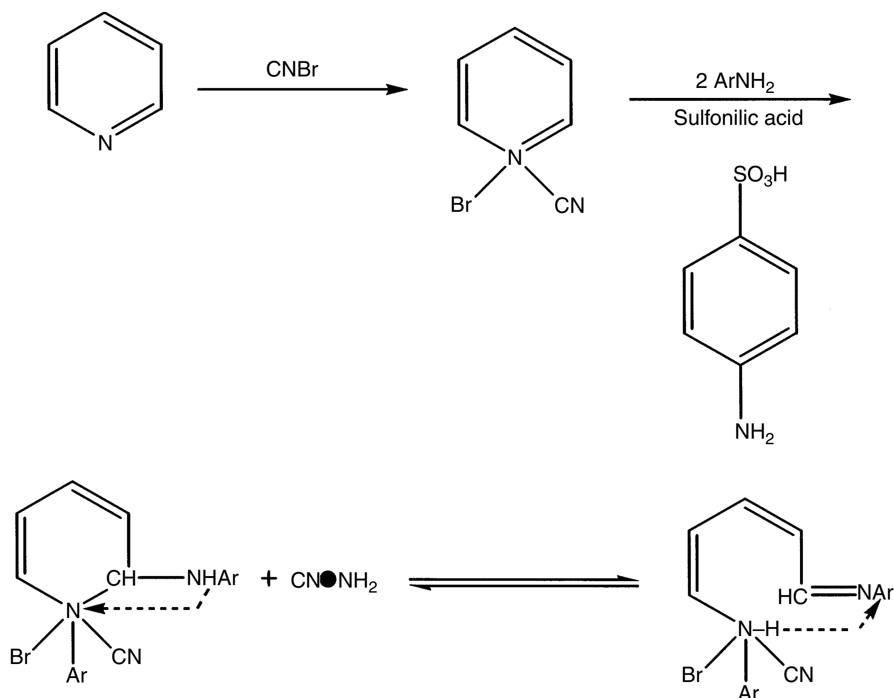

Figure 8.6 Colorimetric reaction for the determination of niacin. (Reproduced from Eitenmiller, R. R. and DeSouza, S., Niacin, In *Methods of Vitamin Assay*, 4th ed., Augustin, J., Klein, B. P., Becker, D. A., and Venugopal, P. B., Eds., John Wiley and Sons, New York, 1985, chap. 15. With permission.)

3. *Cereal products*: Add 1.5 g $Ca(OH)_2$ to each of six 250 mL Erlenmeyer flasks. Add 0, 5, 10, 15, 20, and 25 mL of 10 mg niacin per mL standard solution to each flask for standard curve preparation. Weigh approximately 2.5 g sample into another flask containing 1.5 g $Ca(OH)_2$. Add water to each flask so that volume is approximately 90 mL, mix, and autoclave at 15 psi for 2 h. Mix while hot, cool to 40°C, and transfer to 100 mL volumetric flask. Dilute to volume with water. Extracts can be stored, refrigerated, at this point.

Transfer approximately 50 mL from each flask to centrifuge tubes and place in ice bath for 15 min or in refrigerator for at least 2 h. Centrifuge and pipette 20 mL of supernatant from each tube into separate centrifuge tubes containing 8 g of $(NH_4)_2SO_4$ and 2 mL phosphate buffer, pH 8.0. Dissolve by shaking and warm to 55–60°C. Clarify by centrifugation or filtration.

Determinative steps of the König reaction are provided in Part C of Method 961.14.[47]

Note: The König reaction involves the use of 10% cyanogen bromide solution. All determinative steps should be completed under an approved hood. Do not breathe vapors, and, if solution comes into contact with skin, wash immediately with water.

AOAC Official Method 975.41, Niacin and Niacinamide in Cereal Products, Automated Method, Official Methods of Analysis of *AOAC Int*ernational, 45.1.11

Method 961.14 was automated for the determination of niacin in cereals.[49] The automated method uses inline dialysis for the last clarification step, replacing $(NH_4)_2SO_4$ precipitation. One drop of Brij-35 is added to 100 mL of the diluted extract to avoid bubble

formation in the autoanalyzer tubing. The method was based on the Technicon AutoAnalyzer II system.

AOAC Official Method 981.16, Niacin and Niacinamide in Foods, Drugs, and Feeds, Automated Method Official Methods of Analysis of *AOAC Int*ernational, 45.1.12, American Association of *Cereal Chem*ists—Method 86–52

Method 961.14 was further modified and collaborated for all products other than cereals.[50] Collaborative studies showed that Method 975.41 and Method 981.16 are more precise than the manual procedure (Method 961.14). Also, labor and analysis time are decreased owing to greater sample throughout.

AOAC Official Method 968.32, Niacinamide in Multivitamin Preparations, Spectrophotometric Method, Official Methods of Analysis of *AOAC Int*ernational, 45.1.13

Method 968.32 uses the König reaction to quantitate nicotinamide. Nicotinic acid does not interfere unless present at much higher concentrations than nicotinamide.

Note: Collaborated *AOAC Int*ernational methods using colorimetric procedures provide data that closely compare to microbiological analysis by *Lactobacillus plantarum*.

8.3.2 Microbiological

Microbiological assay of the niacin content in biological samples continues to be an important method worldwide. This, in part, is owing to selectivity and sensitivity problems still influencing the use of LC methods for niacin assay of complex matrices. In addition, microbiological assay of niacin is a well-accepted assay that is quite simple to conduct in the vitamin analysis laboratory.

Lactobacillus plantarum, *Lactobacillus mesenteroides*, and the protozoan, *Tetrahymena thermophila* (pyriformis) have been used for niacin analysis. *L. plantarum* is the primary assay organism. It responds equally on a molar basis to nicotinic acid, nicotinamide, and NAD. Assay of niacin by *L. plantarum* assay is a reliable measure of the total niacin in biologicals and pharmaceuticals. *L. mesenteroides* responds only to nicotinic acid.[63] *T. thermophila* responds equally to nicotinic acid and nicotinamide; however, studies have shown nonadditive responses for the two niacin forms.[64] For analysis of NMN in urine, only *L. plantarum* has growth response. Drift (growth stimulation or inhibition) is seldom encountered with the use of *L. plantarum*. The bacteria is not affected by free fatty acids in the growth media.[65]

*AOAC Int*ernational microbiological methods for niacin use *L. plantarum* ATCC No. 8014. Summaries of the regulatory methods were provided by Eitenmiller and DeSouza[33] and Ball.[34]

AOAC Official Method 944.13, Niacin and Niacinamide (Nicotinic Acid and Nicotinamide) in Vitamin Preparations, Microbiological Method, Official Methods of Analysis of *AOAC Int*ernational, 45.2.04.

Method 944.13 was recommended for application to all foods by the AOAC Task Force on Methods for Nutrition Labeling.[66] Samples are extracted by autoclaving for 30 min in 1.0 N H_2SO_4. After autoclaving, proteins are removed by precipitation at pH 4.5. *AOAC Int*ernational also provides Method 985.34 (Chapter 50.1.19) "Niacin and Niacinamide (Nicotinic Acid and Nicotinamide) in Ready-To-Feed Milk-Based Infant Formula." Method 985.34 is similar to Method 944.13.

Use of 1 N H_2SO_4 hydrolysis for extraction of niacin from cereals should be approached with caution. Method 985.34 is stated to be applicable to cereals; however, it is generally accepted that alkaline hydrolysis must be used to free niacin from the documented bound forms in cereals (Section 8.2.3). The $Ca(OH)_2$ procedure given in AOAC Method 961.14 provides suitable extracts for microbiological analysis. Other, more simple, alkaline digestions

are available that have been used in conjunction with LC analysis.[67–69] These methods are summarized in Table 8.7.

Modified microbiological methods, as opposed to the use of traditional microassay techniques, are available. Guilarte and Pravlik[70] used *Kloeckera apiculata* (brevis) ATCC No. 9774 and radiometric techniques to assay niacin in blood and food. The yeast responded equally to nicotinic acid and nicotinamide, but did not respond to nicotinuric acid, trigonelline, NMN, 2-pyridone, picolinic acid, and quinolinic acid. The method consisted of growing the yeast in media containing L-[1-^{14}C] methionine as substrate with measurement of $^{14}CO_2$. The assay was precise and specific, but the method has not been used by other investigators.

A highly automated microplate method was developed by Solve et al.[71] with *L. plantarum*. The procedure was conducted using 48- and 96-well microplates, video digital-image processing, and an ELISA reader. The method reduced material and time expenditures compared to conventional microbiological assay. Digital imaging was an efficient approach to turbidity measurement. Coefficients of variation ranged from 3.7% to 4.8% for the 48-well and from 16.7% to 25.8% for the 96-well plate assay. The detection limit was 0.5 ng niacin per well. The authors concluded that the 48-well microplate assay had advantages of automation, speed, and minimum reagent costs. Results compared favorably to conventional approaches.

8.3.3 Advances in the analysis of niacin

8.3.3.1 Capillary electrophoresis, chemiluminescence, and mass spectrometry

Capillary electrophoresis (CE) has rapidly developed as an analytical approach for quantitating total niacin in biologicals, foods, and beverages. Methodology reviews through 2001 have been prepared by Iwaki et al.[72] and Trenerry.[73] A summary of recent published methods is given in Table 8.6.[74–86] These published methods cover a broad group of matrices encompassing biologicals, supplements, and foods. Some methods based on GC,[87] chemiluminescence,[88–90] and mass spectrometry (MS)[91–94] are available. With the increasing reliance of analytical biochemists on LC-MS methods, its usefulness for niacin assay will undoubtedly be further defined. Recent LC methods using MS detection are discussed in Section 8.3.3.2.

8.3.3.2 High performance liquid chromatography

Use of LC methods for the measurement of niacin in biological samples has been hindered somewhat by the necessity to use UV detection. While UV detection is suitable for high concentration vitamin premixes and concentrates and for some fortified foods, it lacks specificity and often produces chromatograms with unresolved interferences. Methods for biological samples based on UV detection usually include cleanup procedures to remove interfering compounds. Useful reviews of LC methodology include Ball,[34] Shibata and Taguchi,[35] Eitenmiller and Landen,[36] Lumley,[44] and Iwaki et al.[72] Other, more general, but useful reviews include Finglas and Faulks[95] and Rizzolo and Polesello.[96] LC and LC-MS methods for the analysis of niacin in food and biological samples are summarized in Table 8.6.[68,71,97–120] Postcolumn[105] and precolumn[114] König reaction derivatization and postcolumn photoreaction to convert niacin compounds to fluorescent derivatives[105,114] have been successfully applied to increase specificity and sensitivity of LC-based assays as opposed to UV detection. As noted in Table 8.7, beginning in 2005, LC-MS methods are adding a new and improved capability to analysis of niacin and its metabolites.

8.3.3.2.1 Extraction procedures for analysis of niacin by LC. Both acid and alkaline hydrolyses are used as the initial step in niacin extraction procedures. HCl and H_2SO_4, and NaOH or $Ca(OH)_2$ are the common acids or bases. Details of acid and alkaline hydrolysis procedures are detailed in Table 8.7. Acid hydrolysis will not completely liberate bound niacin forms (Section 8.2.3), and is often used to assay "available" niacin. Alkaline

Table 8.6 Applications of Capillary Electrophoresis Gas Chromatography, Chemiluminescence and Gas or Liquid Chromatography-Mass Spectrometry to the Analysis of Niacin

Matrix	Description	Accuracy/Precision	References
Capillary Electrophoresis			
Injectables, Supplements	MECC 80 cm × 100 μm, 0.02 M phosphate, 75 μA, 254 nm	%RSD ≤ 2.1	*J. Chromatogr.*, 447, 133, 1988[74]
Supplements	CZE 48.5 cm × 50 μm, 0.02 M phosphate, pH 7.0, 20 kV, 215 nm	%RSD—1.6–1.7	*J. Chromatogr. A*, 652, 495, 1993[75]
Supplements	MECC 70 cm × 100 μm, 50 μM Na borate, 22.5 mM SDS, 10% MeOH, pH 8.0, 16 kV, 214 nm	%CV <5	*Electrophoresis*, 15, 1147, 1999[76]
Cereals, meats, and other foods	CZE 75 cm × 75 μm, 0.02 M phosphate, pH 7.0, 20 kV, 254 nm	%CV—1.0	*Food Chem.*, 60, 667, 1997[77]
Yeast spread	CZE 75 cm × 75 μm, 0.02 M vorate—0.02 M phosphate (1:1), pH 9.2, 254 nm	%CV < 4.3	*Food Chem.*, 58, 185, 1997[78]
Supplements	CZE, MECC 48.5 cm × 50 μm, 0.05 M borate (add 25 mM SDS for MECC), pH 8.5, 25 kV, 225 nm	$\%RSD_r$ <6.0 $\%RSD_{int}$—9.1	*J. Pharm. Biomed. Anal.*, 15, 1113, 1997[79]
Foods	CZE 64.5 cm × 50 μm, 0.02 phosphate, 25 kV, 254 nm	%Recovery—84–120	*Food Chem.*, 65, 263, 1998[80]
Legumes	CZE 70 cm × 75 μm, 20 μM borate, 15 mM SDS, 20% IPA, 30 kV, 254 nm	%Recovery >97	*J. High Resol. Chromatogr.*, 21, 81, 1998[81]
Extracellular fluids	CZE 47 cm × 50 μm, 150 mM NaCl, 5 mM Tris-HCl plus 2 mM TTAB, pH 7.4, −10 kV, 214 nm	—	*Electrophoresis*, 20, 2111, 1999[82]
Supplements	CZE, MECC 48.5 cm × 50 μm, 0.05 M borate, pH 8.5 (add 25 mM SDS for MECC) 25 kV, 225 nm	$\%RSD_r$ CZE—1.0–1.8 MECC—0.5–1.6 $\%RSD_{int}$ CZE—1.2–3.0 MECC—1.2–2.6	*J. Chromatogr. A*, 853, 391, 1999[83]
Supplements	CZE, MECC 48.5 cm x 50 μm, 20 mM phosphate-borate, pH 9.0 (Add 50 mM SDS for MECC) 20 kV, 215 nm	$\%RSD_{r\,(migration)}$ MECC—0.59 (NAM) and 0.99 (NA)	*Anal. Chim. Acta*, 396, 55, 1999[84]
Legumes	MECC 71 cm x 75 μm, 20 mM borate, 15 mM SDS, 20% IPA, pH 9.2, 30 kV, 254 nm	—	*Electrophoresis*, 22, 1479, 2001[85]
Propolis	MECC 55 cm x 50 μm, 30 μM borate, 12 mM SDS, 5% (v/v) EtOH, pH 9.0, 14 kV, 214 nm	$\%RSD_r$—2.12	*Fitoerapia*, 75, 267, 2004[86]

Continued

Table 8.6 (Continued)

Matrix	Description	Accuracy/Precision	References
Gas Chromatography			
Supplements, Tonic drinks	CP-Sil 8 CB (30 m x 0.53 mm) Direct injection, FID, H_2 at 30 mL min^{-1}, Carrier gas, N_2 at 5 mL min^{-1}, Injection port - 280°C, Detector - 300°C, 110°C (1.5 min) to 190° at 8°C min^{-1} C Hold 1 min, to 290°C at 40°C min^{-1}	%Recovery—93–108 %CV < 9.8	*J. Food Drug Anal.*, 8, 113, 2000[87]
Chemiluminescence			
NADH	Imaging fiber electrode chemical sensor Distal gold tip, fiber coated with Nafion doped with $Ru(bpy)_3^{2+}$, $Ru(bpy)_3^{2+}$ oxidized to $Ru(bpy)_3^{3+}$	—	*Electroanalysis*, 13, 1287, 2001[88]
Food, supplements	Formation of a luminescent chelate between terbium (III) and nicotinic acid, stopped-flow, Ex λ = 260, Em λ = 542	DL—0.24–0.27 μM %Recovery—91.5–106.1	*Anal. Chim. Acta*, 441, 249, 2001[89]
NADH	Inhibitory effect of NADH on luminol-potassium hexacyanoferrate (III) chemiluminescence	%RSD—1.2 DL—2.0×10^{-8} mol L^{-1}	*Talanta*, 57, 961, 2002[90]
Gas Chromatography/Mass Spectrometry			
Tissue extracts, culture media	GC/Electron-capture Negative Ion MS	$QL_{on\text{-}column}$ < 1 fmol	*Anal. Biochem.*, 301, 21, 2002[91]
Liquid Chromatography/Mass Spectrometry			
Plasma, urine (niacin and six metabolites)	LC-MS/MS High-flow gradient chromatography on Hypersil silica, ESI	%RSD Urine NA—6.4–7.5 NAM—6.2–8.4 Plasmic NA—5.3–9.6 NAM—4.8–10	*Chromatographia*, 58, 723, 2003[92]
Pasta	LC-MS/Electrospray MS (ESI) and atmospheric pressure chemical ionization (APCI)	DL—Low μg L^{-1} Precision—0.2–7.4 %	*Anal. Chim. Acta*, 531, 87, 2005[93]
Human plasma	LC-MS ESI and select ion monitoring (SIM)	QL—50 ng mL^{-1} Precision and accuracy %RSD < 15 %	*J. Pharm. Biomed. Anal*, 36, 1045, 2005[94]

Table 8.7 Selected LC and LC-MS Methods for the Analysis of Niacin

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Plasma, urine/ nicotinamide	Plasma Mix 1 mL with 10 μL isonicotinamide (IS), 400 mg/L. Add 10 μL water. Vortex Urine Mix 1 mL with 10 mL isonicotinamide (IS), 5000 mg/L. Add 10 μL water. Vortex Clean-up – Sep-Pak C_{18}	μBondapak C_{18}, 30 cm × 4 mm, or LiChrosorb RP-18, 10 μm, 25 cm × 4 mm Mobile phase—isocratic Dissolve 4.446 g sodium dioctylsulfosuccinate in 1450 mL water Adjust pH to 2.5 with formic acid Add 1050 mL MeOH Flow rate—2.0 mL min^{-1}	254 nm	QL (mg L^{-1}) 0.1 for plasma 1.0 for urine %Recovery —91.8–92.4	*J. Chromatogr.*, 221, 161, 1980[97]
Various foods/niacin	Acid Hydrolysis. Autoclave 1–5 g in 60 mL 0.1M H_2SO_4, 1 h cool, digest w/Diastase®, 45°C, 3 h, cool, dilute w/water to 100 mL, filter Alkaline Hydrolysis - Add 80 mL water and 10 mL $Ca(OH)_2$ suspension (1 g/10 mL water), heat, steambath, 30 min, autoclave 30 min, dilute to 100 mL w/water. Refrigerate, overnight, centrifuge	Nucleosil 5 C_{18}, 15cm x 4.6 mm and Nucleosil SB anion-exchange. Column switching Mobile phase A. mix 5.7 mL HAC w/800 mL water adjust pH to 3.0 w/NaOH, dilute to 100 mL w/water B. A : MeOH (5:95) C. mix 22.8 mL HAC w/800 mL water, adjust pH to 3.0 w/NaOH, dilute to 1000 mL w/water Column switching A to B to C to A over 55 min	254 nm Also, assayed micro-biologically.	QL—0.5 mg $100g^{-1}$ %CV—6.6 %Recovery—100	*J. Agric. Food Chem.*, 32, 304, 1984[68]
Meats/nicotinic acid, Nicotinamide	Add 30 mL water to 5 g sample homogenize 10 min, cool, dilute to 50 mL, and filter, 0.45 μm	Partisil SCX 10 μm, 25 cm × 4.6 mm, cation-exchange. Mobile phase—isocratic 50 mM phosphate buffer, pH 3.0. Flow rate—1 mL min^{-1}, 25 °C	260 nm	DL (ng) on-column Nicotinic acid—2 Nicotinamide—4 %Recovery— 98–100	*J. Chromatogr.* 457, 403, 1988[98]
Urine/nicotinamide metabolites	To 1 mL urine, add 1.2 g K_2CO_3. Add isonicotinamide (IS). Cool on ice. Add 5 mL Et_2O, mix. Centrifuge. Remove Et_2O layer (3×). Dissolve residue in water. Filter.	7-ODS-L, 7 μm, 25 cm x 4.6 mm Mobile Phase—isocratic 10 mM KH_2PO_4:MeCN (96:4), pH 3.0 Flow rate—1 mL min^{-1}	260 nm	DL (on-column)— 304-1220 pg %Recovery—97.9	*J. Chromatogr.*, 424, 23, 1988[99]

Continued

Table 8.7 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Feeds/Nicotinamide	Add 10 mL 0.2M HCl to 2g sample, stir, 10 min, centrifuge, filter, 0.5 μm	A. RP-18 5 μm, 25 cm x 4.6 mm, and B. Nucleosil 5 SA anion-exchange, 25 cm x 4.6 mm. Column switching Mobile Phase Eluent A—0.01 M KH_2PO_4 Eluent B—0.01 M KH_2PO_4:MeCN (60:40)	264 nm	DL—0.5 ppm QL—2 ppm	*J. Chromatogr.* 502, 79, 1990[100]
Various foods/Nicotinic acid	Add 4 mL 40% NaOH and 4 mL water to 5-10 g sample, heat, steambath, 30 min. Neutralize w/25% HCl, add same volume of MeOH. Filter, rinse residue w/50% MeOH, dilute to 100 mL w/50% MeOH. Evaporate 30 mL aliquot to dryness, dissolve in 20 mL water. Clean-up - AGI-X8 anion-exchange and IC-SPM cation-exchange	Asahipak NH2P-50 5 μm, 25 cm x 4.6 mm Mobile phase—MeCN:water (60 : 40) containing 0.075 M NaOAC	261 nm	%Recovery—91-94	*J. Chromatogr.* 588, 171, 1991[101]
Legumes, meat/Nicotinic acid	Add 30 mL 0.1 N and 1 mL 6 N HCl to 1-10 g sample, autoclave, 15 min. Adjust pH to 4.0-4.5 w/2 N NaOAC, add 5 mL of 6% Takadiastase®, digest at 48 °C, 3 h, filter, dilute to 100 mL w/water Clean up – Dowex 1-X8	μBondapak C_{18}, 10 μm, 30 cm x 3.9 mm or Spherisorb ODS 2 10 μm, 30 cm x 3.9 mm Mobile phase—isocratic 0.005 M TBAB in MeOH : 0.01 M NaOAC (1:9) pH 4.72 Flow rate—1.5 mL min^{-1}	254 nm	—	*J. Agric. Food Chem.* 39, 116, 1991[102]
Fortified foods/Nicotinic acid	Weigh sample containing 100 μg niacin into 500 mL Phillips beaker, add 50 mL water and 6 mL H_2SO_4 (1:1), mix. Autoclave, 45 min, cool, adjust pH to 6.0 – 6.5 w/7.5 N NaOH, dilute to 100 mL w/water Clean up - Florisil	PRP-X100, 25 cm x 4.1 mm Mobile phase—isocratic 20 mL HAC diluted to 1 L w/water	254 nm	QL—0.11 μg mL^{-1} %CV—2.4-2.9 %Recovery—99.8	*J. AOAC Int.* 76, 390, 1993[103]

Urine/Nicotinic acid, nicotinuric acid	Dilute urine (300 µL) with 600 µL water. Add 6-methyl nictonic acid (IS) (20 µg/mL) Clean-up – Bond Elut SCX	Invertisil ODS-2, 5 µm, 25 cm x 4.6 mm Mobile Phase—isocratic 10 mM K_2HPO_4 containing 5 µM TBAP, pH 7.0 : MeCN (90 : 10) Flow rate—1 mL min^{-1}, 35°C	254 nm	QL—5 µL mL^{-1} %CV—8.1-8.8	*J. Chromatogr. B*,, 661, 154, 1994[104]
Biologicals/Nicotinic acid, nicotinamide	Homogenize in saline. Add 18 mL acetone to 3 mL, homogenate. Centrifugate. Filter 500 mL of upper aqueous phase, 0.45 µm. Add [^3H] nicotinic acid for recovery study.	Hypersil ODS, 5 µm, 25 cm x 4.6 mm Mobile Phase—gradient A. 100% MeOH B. 5 mM TBAP, pH 7.0 0-10 min, A : B (90 : 10) to (30 : 70) Flow rate—1.2 mL min^{-1}	Post-column derivatization using König reaction 410 nm	DL (on-column)—12-21 pmol %RSD < 5 %Recovery—92.4	*J. Chromatogr. B*, 665, 71, 1995[105]
Cereals, meats, foods/ Nicotinic acid	Add 0.5 g $Ca(OH)_2$ and 20 mL water to 1 g sample, heat at 121°C, 2 h, cool, centrifuge, adjust 15 mL aliquot to pH 7.0 with 10 % oxalic acid followed by 1 %, dilute to 25 mL with water Clean-up – C_{18} Sep-Pac Vac Cartridge (500 mg) in Series with SCX column (500 mg)	C_8 Nova-PAK Radial PAK, 10 cm x 8 mm Mobile Phase MeOH/water (15 : 85) containing 0.005 M PICA	254 nm	%Recovery—92-110	*Food Chem.*, 60, 667, 1997;[77] *J. Food Compos. Anal.*, 16, 93, 2003[106]
Foods/Niacin	Add 0.1 M HCl (30 mL) to 1-5 g finely ground sample. Heat in water bath at 100 °C for 1 h. Cool, dilute to 50 mL w/water. Filter. Add 5 mL 5 M NaOH to 25 mL aliquot, autoclave at 120 °C for 1 h. Cool, adjust pH to 4.5 and dilute to 50 mL w/water. Filter solution first through filter paper then through 0.45 µm cellulose nitrate filter. Inject 20 µL	Lichrospher 100 RP 18, 25 cm x 5 mm Post-column derivatization photochemical reaction carried out in a PTFE tube (10 m x 0.5 mm i.d.). Mobile phase—isocratic 0.07 M KH_2PO_4/0.075 M H_2O_2/5.10^{-6} M $CuSO_4$ Flow rate—1.0 mL min^{-1}	Fluorescence Ex λ = 322 Em λ = 380	QL—0.2 µg g^{-1} %Recovery—90-107 %CV < 4	*Food Chem.* 65, 129, 1999[107]

Continued

Table 8.7 (Continued)

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Cooked Sausages/ Nicotinic acid, nicotinamide	Weigh 10 g finely ground sample into 50 mL beaker, add 30 mL water, homogenize for 2 min. Transfer to a 100 mL volumetric flask and dilute to volume w/water. Centrifuge at 3000 rpm for 5 min, filter upper solution. Collect 20 mL filtered extract to 25 mL volumetric flask. Add 1 mL saturated $ZnSO_4$ solution and 1 mL 1 N NaOH. Dilute w/water to the volume, mix, stand, 30 min. Filter (No. 1305 filter paper and 0.45 µm Millipore filter). Inject 10 µL	Spherisorb ODS-2, 5 µm 25 cm x 4 mm Mobile phase—isocratic 5 mM Heptanesulfonic acid (pH 3.3) : ACN (75 : 25) Flow rate—0.65 mL min^{-1}, 35 °C	261 nm	DL—0.3 mg $100g^{-1}$ %Recovery >92	*J. Agric. Food Chem*. 48, 3392, 2000[108]
Infant formula/ Niacin	Accurately weigh sample containing 100 – 200 µg niacin in 150 mL fleaker, add 8 mL demineralized water and 2 mL (1+1) H_2SO_4, mix. Cover w/75 mm watch glass. Autoclave at 121-123 °C for 45 min, cool. Adjust pH to 6.5 w/7.5 N NaOH, then immediately adjust to pH ≤ 1.0 w/H_2SO_4. Filter. Clean up – ArSCX-SPE or sulfonated Florisil-SPE. Inject 100 µL	PRP-X100 anion-exchange, 25 cm x 4.1 mm Mobile phase 0.1 M NaAC—HAC, pH 4.0-4.2 Flow rate—1.5 mL min^{-1}	260 nm	DL—0.2 µg mL^{-1} QL—0.7 µg mL^{-1} %Recovery >91 by ArSCX-SPE >85 by sulfonated Florisil-SPE	J. *AOAC Int*. 82, 128, 1999;[109] J. *AOAC Int*. 84, 789, 2001;[110] J. *AOAC Int*. 85, 654, 2002[111]
Foods/Nicotinic acid, Nicotinamide	Accurately weigh 5 g finely ground sample into a conical flask. Add 50 mL 50 mM NaAC (pH 4.5), incubate at 37 °C for 18 h in the presence of 200 µL NADase. Dilute to 100 mL w/water. Filter (filter paper and 0.45 µm Millipore filter). Inject 20 or 100 µL	Uptishpere C_{18} HDO column, 15 cm x 4.6 mm, 5 µm Post-column photochemical derivatization carried out in a PTFE tube (5 m x 0.5 mm i.d.). Mobile phase—isocratic 0.07 M KH_2PO_4/0.075 M H_2O_2/5.10^{-6} M $CuSO_4$ Flow rate—1.0 mL min^{-1}	Fluorescence Ex λ = 322 Em λ = 380	—	*Food Chem*. 78, 129, 2002[112]

Pharmaceuticals/ Nicotinamide, 4-aminobenzoic acid	Dissolve sample equivalent to 11 mg nicotinamide in 4 mL HAC, add 50 mL MeOH, shake, add 40 mL water, sonicate, dilute with water, filter	µBondapack C_{18}, 30 cm x 4.6 mm, 5 µm Mobile Phase—isocratic MeOH : 0.05 M phosphate (75 : 925), pH 3.6 Flow rate—1.5 mL min^{-1}	254 nm	$\%RSD_{int}$—0.47 DL—2 µg mL^{-1}	*J. Pharm. Biomed. Anal.*, 29, 723, 2002[113]
Serum, urine, supplements/Nicotinic acid, nicotinamide	Supplements Dissolve in MeOH, dilute with 100 mM SDS Serum, urine No sample preparation required	Kromasil C_{18}, 25 cm x 4.4 mm, 5 µm Mobile Phase—isocratic 150 mM SDS/6% (v/v) pentanol with 10 mM NaH_2PO_4, pH 3.0	Precolumn König derivatization, 440 nm	Nicotinic acid $\%RSD_{Intra}$—1.4-3.3 $\%RSD_{Inter}$—1.9-3.6 Nicotinamide $\%RSD_{Intra}$—1.9-2.1 $\%RSD_{Inter}$—1.8-2.3	*Anal. Chim. Acta*, 517, 81, 2004[114]
Fresh and cured pork/ nicotinic acid	Add 25 mL 1N HCl and 5 mL MeOH to 5-10 g homogenized sample, heat at 121°C, 30 mm, cool, precipitate protein with TCA, filter, dilute to 100 mL with water	OmniPac PCX 500, 25 cm x 2 mm Eluent—140 mM HCOOH/15 mM NH_4 COOH/5% MeCN	262 nm	%Recovery—88-95	*Food Chem.*, 92, 373, 2005[115]
Cereals/Nicotinamide	Suspend sample containing 100 – 200 µg nicotinamide in 15 mL 0.6 M TCA, shake, 15 min, centrifuge, 20 min. Filter an aliquot supernatant through 0.45 µm, dilute to 30 mL.	Vydac 201TP C_{18}, 15 cm x 4.6 mm Mobile phase MeOH:H_2O (25:75, 1% formic acid) (adjust pH to 2.7-2.9 w/KOH), add 1.0 g ion-pairing reagent, dioctyl sulfosuccinate, to the mobile phase. Flow rate—0.6 mL min^{-1}	260 nm	%Recovery—100	*Cereal Chem.*. 82, 277, 2005[116]
LC-MS					
Human plasma/niacin, niacinamide, nicotinuric acid	Spike 1.0 mL plasma with 250 ng chinoline-3 carboxylic acid (IS). Add 1 mL 1% formic acid SPE on Isolute SCX, elute with 2 mL MeCN, 2 mL MeOH, 2 mL Hex. Elute analytes with 2 mL MeOH containing 2% ammonia.	Grom Hypersil CPS, 5 µm, 25 cm x 2 mm Mobile phase—isocratic MeCN : MeOH : water : formic acid (700 : 190 : 110 : 1) Flow rate—0.2 mL min^{-1}	LC-MS-ESI positive ionization mode with SIM	%Recovery—86-89 Extensive validation provided	*J. Pharm. Biomed. Anal.*, 36, 1045, 2005[94]

Continued

Table 8.7 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Rabbit plasma/ nicotinic acid, myristyl nicotinate, nicotinamide	Add d-Nia-114, d-NIC and d-NAM in 85:15 (MeOH:water) to MEK (IS spike solution). To 200 µL of plasma, add 1 mL MEK containing the Iss. Hold at ambient temperature for 20 min, vortex. Centrifuge, evaporate 1 mL aliquot. Redissolve in mobile phase	Spherisorb Cyano, 5 µm, 15 cm x 4.6 mm Mobile phase—isocratic MeCN : water : formic acid (56 : 44 : 0.1) Flow rate—1 mL min^{-1}	LC-MS/MS-ESI positive ion mode Collision induced dissociation with MRM	Niacin $\%RSD_{Intra}$—2–12 $\%RSD_{Inter}$—4.6–15	*J. Chromatogr. B*, 829, 123, 2005[117]
Plasma, urine/NAD metabolites	Add 2-chloroadenosine (IS). Deproteinize with 10% TCA. Extract with Et_2O	Hypersil C_{18}-BDS, 3 µm, 15 cm x 2.0 mm Mobile phase—gradient Solvent A—10 mM nonafluoropentanoic acid in water Solvent B—MeCN 0-60% B in 12 min Flow rate—0.2 mL min^{-1}	LC-MS-ESI positive SIM mode or LC-MS/MS positive ion mode	%Recovery—75-95 %CV < 10 (repeat injections) > 20 (between day)	*Nucleos. Nucleot. Nucl. Acids*, 25, 1245, 2006[118]
Plasma/nicotinic acid	Extract using Strata-X-C 33 µm cation mixed-mode polymer 96-well SPE plates. Add d_4-nicotinic acid (IS)	Oasis MCX, 5 µm, 10 cm x 2.1 mm Mobile phase—gradient A—1% formic acid B—MeCN : water (50 : 50) 0% B for 2 min 0% B to 100% B in 6 min Maintain 3 min Flow rate—500 µL min^{-1}	LC-MS/MS w/ SRM	%CV—7.8-24.1 QL—0.05 µg mL^{-1}	*J. Pharm. Biomed. Anal.*, 41, 510, 2006[119]
Cells/NAD^+, NAAD, NMN/NAMN, NAM, NA, ADPR 5′-AMP	Collect blood into heparin, centrifuge. Add 10 volumes of 0.5 N perchloric acid containing 0.1 µM 2-chloro-adenosine (IS) to 1 volume of packed cells, keep on ice for 10 min, centrifuge	Atlantis dC_{18}, 3 µm, 15 cm x 2.1 mm Mobile phase—gradient A—5 mM NH_4 formate B—MeOH 100% A to 70% B in 10 min Hold 5 min	LC-MS/MS-ESI Positive ion mode	%Recovery—84-112 %CV—1-5.9	*Anal. Biochem.*, 352, 282, 2006[120]

hydrolysis liberates most bound forms and provides a measure of total niacin. Choice of extraction parameters depends on the following factors:

1. If total niacin is to be determined in cereals, alkaline hydrolysis must be used.
2. Fortified forms of niacin can be extracted by acid, water, or ethanol extractions, usually without autoclaving. Hydrolysis is necessary for extraction of complex food and clinical samples.
3. Blood, serum, and urine do not require hydrolysis for efficient niacin extraction.
4. Acid hydrolysis can produce partial conversion of nicotinamide to nicotinic acid.[121]
5. Alkaline hydrolysis converts nicotinamide to nicotinic acid, resulting in simpler chromatography of fortified foods that might contain both forms.
6. Biological samples usually require extract purification following initial extractions. Cleanup is necessary owing to a lack of specificity of UV detection.

The most common extract purification approaches use ion-exchange chromatography with anion-exchange resin or solid phase C_{18} extraction columns. Chase et al.[103] used Florisil open-column chromatography to successfully purify H_2SO_4 extracts of fortified foods. Detection at 254 nm following chromatography on PRP-X100 anion exchange resin gave interference-free chromatograms. The Chase et al.[103] procedure is detailed in Section 8.4. LaCroix and coworkers[109–111,122] applied an aromatic sulfonic acid solid-phase extraction (SPE) column cleanup procedure to the sulfuric acid digestion used in *AOAC Int*ernational Method 944.13 that is recommended for vitamin preparations and foods.[66] The procedure, which is detailed in Section 8.5, successfully removed interferences often present in digests that are quantified by LC methods using UV detection. The SPE-treated extracts were chromatographed on a PRP-X100 anion-exchange column with detection at 260 nm. The method was peer-verified for assay of infant formula and given the designation PVM1:2000 by *AOAC Int*ernational.[110,111] Comparison of the method to other commonly used extraction procedures used for LC analysis of niacin showed that the SPE method provided better spectral matches than a procedure based on trichloroacetic acid extraction[123] (TAC) and one using metaphosphoric acid extraction.[124] LaCroix et al.[122] adapted the TCA extraction method[123] to the LC analysis of unbound nicotinamide in cereals. This method is a simple and fast method for assay of nicotinamide added to cereals through fortification.

Windahl et al.[80] used acid extraction with 0.8 M sulfuric acid and autoclaving for 2 h to free niacin from raw and cooked meat and fish. The autoclave step was followed by C_{18} and cation exchange SPE treatments. The extracts were sufficiently clean for CE or LC analysis with UV detection at 254 nm. However, the extraction was not recommended for most other food classes. For others, alkali extraction was recommended.

8.3.3.2.2 Chromatography parameters

8.3.3.2.2.1 Supports and mobile phases. Supports and mobile phases used in recently developed methods for niacin analysis are given in Table 8.7. Poor resolution of niacin from interfering components in biological extracts that absorb at UV wavelengths used for niacin detection has led investigators to develop quite complex chromatographic systems in addition to the use of extensive extract purification methods. Most methods capable of resolving niacin use anion– and cation-exchange resins or reversed-phase chromatography on C_{18}. van Niekerk et al.[68] introduced a complex column switching technique to resolve niacin from eluting UV absorbing interferences. The method used C_{18} reversed-phase and anion-exchange chromatography on Nucleosil 5-SB support. Mobile phases used with the system were based upon glacial acetic acid:water combinations and the cycle was completed in 55 min. Other investigators have effectively used column switching on C_{18} and anion-exchange columns.[100] Ion-pair chromatography can improve resolution of niacin from interfering peaks, but ion-pairing reagents have not been universally used. Commonly

used ion-pair reagents include tert-butylammonium hydroxide (TBAH), tetrabutylammonium phosphate (TBAP), sodium dodecylsulfate (SDS), tetrabutylamminoum bromide (TBAB), and sodium dioctylsulfosuccinate.

8.3.3.2.2.2 Detection. UV detection presents a convenient, although nonspecific, detection mode for niacin. Most studies to date utilized absorbance at 254–264 nm. A newer approach to niacin detection after LC resolution uses the chromaphore developed by the König reaction with KCN.[105] The postcolumn derivatization provided extremely clean chromatograms with high specificity for nicotinic acid and nicotinamide. The method is summarized in Section 8.4 and deserves investigation for its general application to biological samples. Capella-Peiró et al.[114] applied the König reaction precolumn to the analysis of nicotinic acid and nicotinamide in pharmaceuticals and biologicals with equally good results. Resolution was obtained on a Kromasil C_{18} column under micellar chromatography. Use of KCN remains an obstacle, but the reagent can be safely handled.

Postcolumn derivatization by UV irradiation in the presence of copper(II) ions and hydroperoxide to convert niacin components to fluorescent derivatives was first introduced by Mawatari et al. [125] for serum analysis. The photochemical derivatization was applied to food analysis by Lahély et al.[107] in 1999. These authors used a precolumn hydrochloric acid hydrolysis to free biologically active forms of niacin from the food matrix and convert niacinamide to nicotinic acid. The postcolumn derivatization was completed in a 10 cm × 0.5 mm i.d. PTFE tube wound around a black light (300–400 nm) with a filter excluding the 254 wavelength. Fluorescence was monitored at Ex $\lambda = 322$ and Em $\lambda = 380$. Ndaw et al.[112] used the procedure to assay the niacin content of a wide variety of foods. In the later paper, nicotinic acid and nicotinamide were assayed following digestion with NAD glycohydrolase (NADase, E.C.3.2.2.5) from *Neurospora crassa*.

As is the case for many other vitamins, assay methodology is rapidly shifting to LC-MS methods that take advantage of the selectivity and sensitivity of MS detection.[94,117–120] Indicative of this work, Pfuhl et al.[94] and Slominska et al.[119] used MS detection to quantify niacin and its metabolites in serum. Pfuhl et al.[94] presented a method with extensive validation for assay of niacin, niacinamide, and nicotinuric acid (Figure 8.7). Slominska et al.[118] quantified niacin, NAD, and several catabolites (Figure 8.8). Both LC-MS and tandem mass spectrometry (LC-MS/MS) have been used in recently published methods.

8.3.3.2.2.3 Internal standards. Internal standards have not been routinely used in LC quantitation of niacin. Isonicotinamide was the internal standard in methods developed for plasma and urine.[44,45] Detection by mass spectrometry offers the advantage of easily incorporating structurally similar internal standards into the analysis with unequivocal detection. LC-MS and LC-MS/MS studies have used chinolin-3-carboxylic acid,[94] deuterated compounds (d_4-nicotinic acid,[119] d_4-niacin-114,[117] and d_4-nicotinamide[117]) and 2-chloroadenosine.[118,120] Each of the LC-MS or LC-MS/MS studies cited from 2005 shows the precision, accuracy, and sensitivity afforded to the analysis of niacin and its metabolites.

8.4 Method protocols

Liquid Chromatographic Analysis of Niacin in Fortified Food Products
J. AOAC Int., 76, 390, 1993[103]

Principle

After initial extraction by autoclaving with H_2SO_4 (1 + 1), the extracts were purified using Florisil open-column chromatography. The amount of niacin was determined by LC and UV detection at 254 nm.

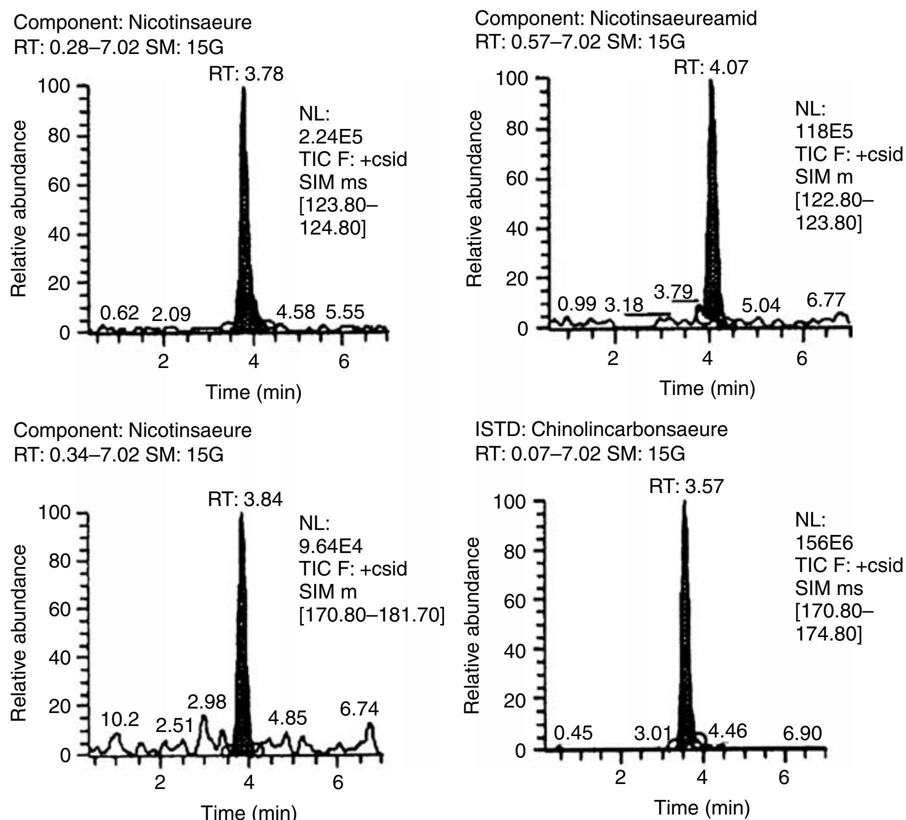

Figure 8.7 SIM-chromatography of a calibration standard of niacin (75 ng mL^{-1}), nicotinamide, nicotinic acid, and chinolin-3-carboxylic acid (IS). (Reproduced from Pfuhl, P., Kärcher, U., Häring, N., Baumeister, A., Tawab, M. A., and Schubert-Zsilavecz, M., *J. Pharm. Biomed. Anal.*, 36, 1045, 2005. With permission.)

Chemicals

- Florisil
- H_2SO_4
- Methanol

Apparatus

- Liquid chromatograph
- UV detector
- Glass columns, 30 cm × 10.5 mm for open-column chromatography

Procedure

Extraction of Standards and Samples

- Weigh sample to contain 100 mg niacin
- Add 150 mL water and 6 M H_2SO_4 (1 + 1)
- Mix well to break up clumps
- Autoclave at 121°C–123°C for 45 min
- Adjust pH to 6.0–6.5 with 7.5 N NaOH

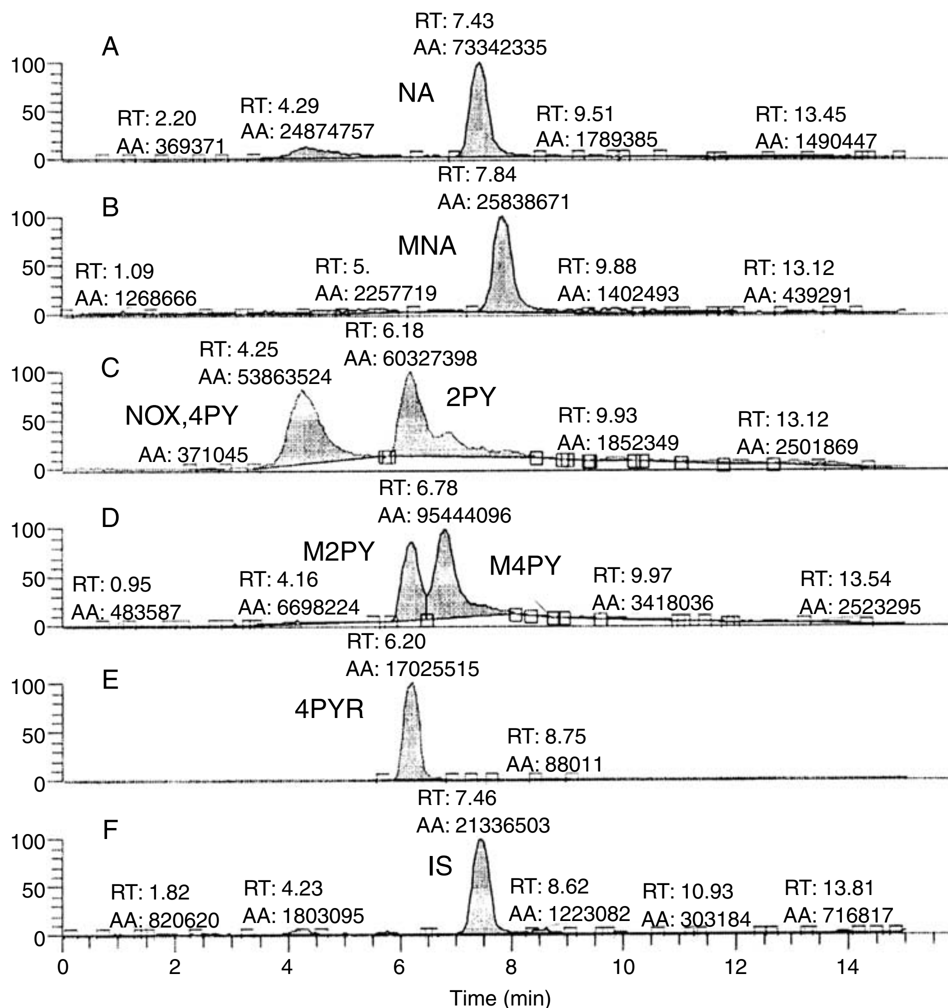

Figure 8.8 Ion chromatogram of the standards of nicotinamide metabolites, nicotinamide (NA), *N*-methylnicotinamide (MNA), nicotinamide *N*-oxide (NOX), *N*-methyl-2-pyridone-5-carboxamide (M2PY), *N*-methyl-4-pyridone-3-carboxamide (M4PY), and 2-chloroadenosine (IS). (Reproduced from Slominska, E. M., Adamski, P., Lipinski, M., Swierczynski, J., and Smolenski, R. T., *Nucleosides Nucleotides Nucleic Acids*, 25, 1245, 2006. With permission.)

- Adjust pH to 4.5 with H_2SO_4 (1 + 1)
- Dilute extract to 100 mL with water
- Filter, Whatman No. 40
- Adjust 20.00 mL aliquot of filtrate to pH 0.5–1.0 with H_2SO_4 (1 + 1)

Open-Column Clean Up

Preparation of Florisil Column

- Add 4 g Florisil to column
- Top with small pledglet of glass wool
- Prewash column with 30 mL methanol, followed by two 15 mL portions of 0.5 N H_2SO_4

Sample Extract

- Transfer extract to Florisil column
- Wash column with two 15 mL portions of 0.5 N H_2SO_4
- Discard effluent
- Elute niacin with 25 mL 0.5 N NaOH into a 50 mL volumetric flask containing 1.0 mL glacial acetic acid
- Dilute to volume with water
- Filter (0.45 mm filter)

Chromatography*

Column	25 cm × 4.1 mm
Stationary phase	PRP* ×100
Mobile phase	glacial acetic acid:water (20:980)
Column temperature	Ambient
Flow rate	1.5 mL min^{-1}
Retention time	8 min
Detection	UV, 254 nm
Calculation	External standard, linear regression: 0.25, 0.48, 0.64, and 0.80 mg mL^{-1}

*Figures 8.9 and 8.10.

High-Performance Liquid Chromatographic Determination of Nicotinic Acid and Nicotinamide in Biological Samples Applying Postcolumn Derivatization Resulting in Bathmochrome Absorption Shifts
J. Chromatogr. B, 665, 71, 1995[105]

Principle

Tissue was homogenized in saline. After addition of acetone, the extract was centrifuged. After chloroform addition, the extract was recentrifuged. The aqueous layer was filtered

Figure 8.9 Chromatogram of a niacin standard solution. (Reproduced with permission from Chase, G. W., Jr., Landen, W. O., Jr., Soliman, A. M., and Eitenmiller, R. R., *J. AOAC Int.*, 76, 390, 1993.)

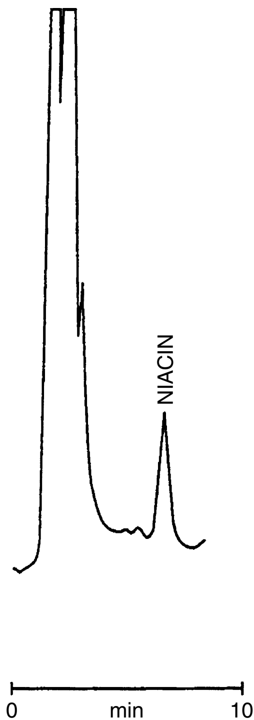

Figure 8.10 Chromatogram of nicotinic acid with a milk-based infant formula. (Reproduced with permission from Chase, G. W., Jr., Landen, W. O., Jr., Soliman, A. M., and Eitenmiller, R. R., *J. AOAC Int.*, 76, 390, 1993.)

and injected. LC analysis used C_{18} support and a linear gradient of tetrabutylammonium phosphate (TBAP) and methanol. Postcolumn derivatization with KCN (König reaction) yielded clean chromatograms at 410 nm.

Chemicals

- Nicotinic acid, nicotinamide
- Chloramin T
- Tris
- Methanol
- Acetone
- Chloroform
- TBAP
- Potassium cyanide

Apparatus

- HPLC, gradient controller
- Visible detector
- Centrifuge
- Solvent metering pump
- Water bath

Procedure

Sample Extraction

- Homogenize tissue in saline
- Add acetone, centrifuge
- Add chloroform to supernatant
- Centrifuge
- Filter aqueous layer

Chromatography*

Column	25 cm × 4.6 mm
Stationary phase	ODS Hypersil, 5 μm
Mobile phase	Linear gradient of 100% methanol and 5 mM TBAP, pH 7.0
Column temperature	Ambient
Flow rate	1.2 mL min^{-1}
Detector	410 nm
Postcolumn derivatization	KCN (Figure 8.11), 60°C
Calculation	External calibration, peak area, regression

*See Figures 8.11 and 8.12.

The Determination of Niacin in Cereals, Meat, and Selected Foods by Capillary Electrophoresis and High Performance Liquid Chromatography
Food Chem., 60, 667, 1997[77]

Principle

Total niacin is extracted by alkaline hydrolysis and concentration and purification on C_{18} and SCX cation exchange columns in series. Capillary electrophoresis on fused silica resolved niacin with detection at 254 nm.

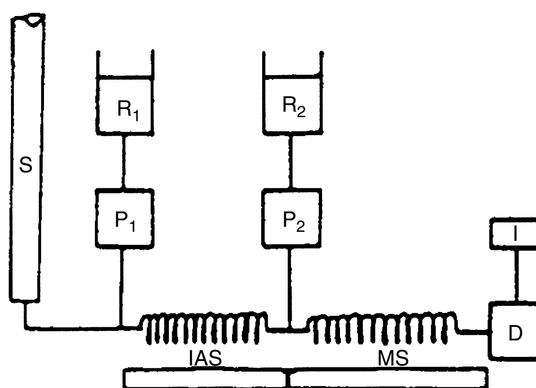

Figure 8.11 Schematic for the chromatography system and postcolumn derivatization of niacin by KCN. 1–2% cloramine T, P_1-pump, R_2-0.25% KCN, 25 mM Tris, 40 mM HCl, P_2-pump, D-detector, I-integrator. Reaction coils are 2 m PTFE, 0.5 mm ID after R_1 delivery and 8 m PTFE, 0.5 mmID after R_2 delivery. Coils are immersed in a 60°C water bath. Flow rate equals 0.5 mL min^{-1}. (Reproduced from Stein, J., Hahn, A., and Rehner, G., *J. Chromatogr. B*, 665, 71, 1995. With permission.)

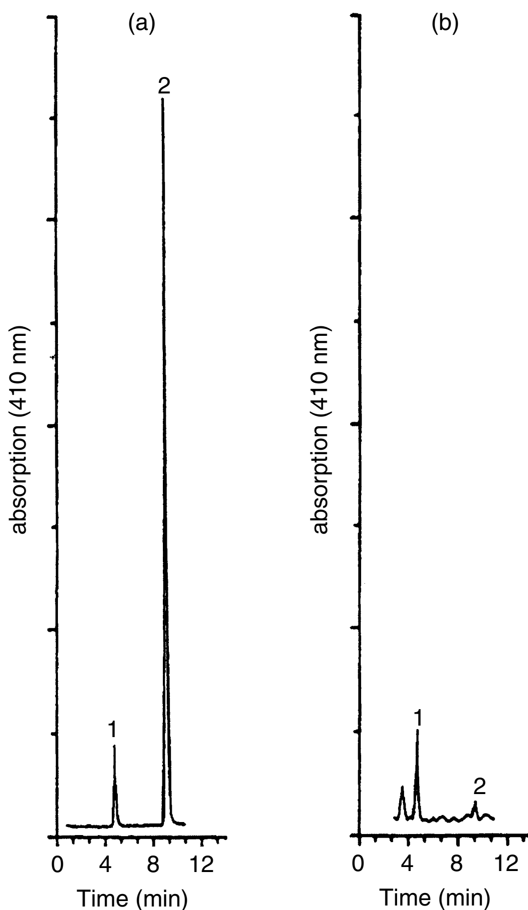

Figure 8.12 Chromatograms of nicotinic acid and nicotinamide after postcolumn derivatization. (a) standards, (b) rat intestinal tissue, Peak 1 = nicotinic acid, Peak 2 = nicotinamide. (Reproduced from Stein, J., Hahn, A., and Rehner, G., *J. Chromatogr. B*, 665, 71, 1995. With permission.)

Chemicals

- Niacin
- Saccharin (IS)
- Disodium hydrogen orthophosphate
- Oxalic acid
- Methanol
- Ammonium hydroxide
- Acetonitrile

Apparatus

- Capillary electrophoresis unit with UV detector
- Sep-Pak C_{18} Vac cartridge
- SCX column (500 mg)
- Cellulose acetate filter assembly (0.8 µm)

Procedure

Extraction

- To 1 g of sample, add 0.75 g calcium hydroxide and 20 mL deionized water.
- Autoclave, 2 h at 121°C.
- Cool, dilute to 50 mL with water.
- Centrifuge.
- To 15 mL aliquot of supernatant, adjust pH to 7.0 with 10% solution of oxalic acid.
- Dilute to 25 mL with water, centrifuge at 0°C.

SPE Cleanup and Concentration

- Condition Sep-Pak Vac C_{18} and SCX columns connected in series with 10 mL methanol followed by 10 mL deionized water.
- Load 10 mL extract onto the C_{18} column.
- Wash columns with 5 mL water.
- Discard C_{18} column.
- Wash SCX column with 5 mL methanol.
- Elute niacin with 5 mL of freshly prepared 2% solution of ammonium hydroxide in methanol.
- Evaporate eluate under nitrogen.
- Dissolve residue in 1 mL of aqueous saccharin (40 mg mL^{-1}).
- Filter, 0.8 µm cellulose acetate.

Capillary Electrophoresis*

Column	75 cm × 75 mm uncoated fused silica
Effective length	50 cm to detection
Buffer	15% acetonitrile and 85% of a 1:1 mixture of 0.02 M potassium hydrogen orthophosphate and 0.02 M disodium hydrogen orthophosphate, pH 7.0
Voltage	+20 kV
Temperature	30°C
Detection	254 nm
Load	Under vacuum

*See Figure 8.13.

Determination of Niacin in Infant Formula by Solid-Phase Extraction and Anion-Exchange Liquid Chromatography

J. AOAC Int., 85, 654, 2002[111]

Principle

After digestion at 121°C with (1 + 1) H_2SO_4, the filter digest is treated by SPE with aromatic sulfonic acid-SPE (ArSCX-SPE). Niacin in the form of nicotinic acid is quantified by LC or an anion-exchange polystyrene-divinyl benzene column with UV detection at 260 nm.

Chemicals

- Nicotinic acid
- Sodium acetate
- Glacial acetic acid
- Methanol
- Demineralized water

Apparatus

- Liquid chromatograph
- Vacuum pump
- UV detector
- Filters (0.45 μm)

Procedure

Extraction of Infant Formula

- Quantitatively transfer powder on liquid to 150 mL beaker with addition of 10 mL water (70–90°C).
- Solubilize and add 2.0 mL (1 + 1) H_2SO_4.
- Autoclave at 121°C for 45 min, cool.
- Adjust to pH 6.5 with 7.5 M NaOH and immediately to pH 1.0 with 1.5 mL (1 + 1) H_2SO_4.
- Filter through prewashed Whatman No. 605 or 588 pleated filter paper into 50 mL screw cap conical centrifuge tube.
- Bring filtrate to 35.0 mL with water.
- Treat standard in a similar manner.

Solid Phase Extraction

- Place ArSCX-SPE column on the vacuum manifold.
- Wash column 3× with 6 mL methanol, followed by 3× wash with 6 mL water.
- Add 3 mL filtrate to column.
- Adjust vacuum to obtain a flow rate of 1.45 mL min^{-1}.
- When all liquid has entered column, wash excess fluid from column with 3.0 mL water, discard eluants.
- Elute niacin with 6.0 mL 0.25 M sodium acetate/0.25 M acetic acid buffer, pH 5.6, repeat with an additional 6.0 mL buffer.
- Bring extracts to volume of 15.0 mL with 0.5 M sodium acetate/acetic acid buffer.

Chromatography

Column	25 cm × 4.6 mm
Stationary phase	PRP-X100 (anion-exchange)
Mobile phase	0.2 M sodium acetate—acetic acid, pH 4.0–4.2
Column temperature	–35°C
Flow rate	1.5 mL min^{-1}
Detector	UV, 260 or 254 nm
Calculation	External calibration

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chapter nine

Vitamin B_6

9.1 Review

In 1934, Gyorgy identified vitamin B_6 as a curative factor for a characteristic dermatitis in rats. The vitamin was isolated in the pure crystalline state in 1938 by several researchers. Gyorgy named the vitamin pyridoxine, which was structurally characterized and synthesized in 1939. The complexity of the vitamin B_6 group was not understood until Snell[1] identified the existence of pyridoxal and pyridoxamine in some of the early historically significant growth studies on the lactic acid bacteria. Human deficiency symptoms were first observed in 1950 by Mueller and Vilter.[2] Marginal vitamin B_6 deficiency is usually associated with other nutritional deficiencies. General symptoms include weakness, sleeplessness, nervous disorders, appetite and growth depression, and various dermatologic disorders. Table 9.1 summarizes deficiency symptoms as presented by Combs.[3] Genetic defects involving vitamin B_6-dependent enzymes, while not diet dependent, produce symptoms that mimic deficiency.[4] Individuals with congenital enzyme deficiencies sometimes respond to high doses of vitamin B_6.

The metabolically active form is pyridoxal-5′-phosphate (PLP). PLP acts as a cofactor in a large number of amino acid transformations, most notable of which is transamination. In general, PLP enzymes act through Schiff base aldimine intermediates with the formation of a resonance stabilized carbanion. Vitamin B_6-dependent enzymes are categorized in Table 9.2. The primary route for PLP catabolism is through oxidation to 4-pyridoxic acid (4-PA), which is excreted in the urine. Pyridoxal (PL) and 4-PA are the primary urinary excretion forms. Concentrations of 4-PA in the urine respond rapidly to dietary intake and are not considered to be a good indicator of status.[5] As recently reviewed by Gibson[5], the three most widely used biochemical tests to assess vitamin B_6 status for the human are the activation coefficient for the erythrocyte aspartate aminotransferase, plasma PLP concentration, and urinary excretion of 4-PA. The plasma PLP concentration is a direct measure of the coenzyme form of vitamin B_6 and is considered the best measure of status. Inadequacy has been defined as levels less than 20 nmol L^{-1}.[6] Methods to measure plasma B_6 levels include the tyrosine apodecarboxylase procedure and liquid chromatography (LC) methods (Section 9.4). Radiometric methods include the conversion of [1-^{14}C] tyrosine to $^{14}CO_2$ by apo-tyrosine decarboxylase and the vitamin B_6 ^{3}H radioenzymatic assay based on the PLP-dependent tyrosine decarboxylase. These methods are used in clinical laboratories for measurement of PLP in plasma.[7–9] Han et al.[10] introduced an enzymatic PLP assay that uses the apo form of the PLP-dependent recombinant enzyme, homocysteine-α-γ-lyase (rHCYase), which does not require radioisotopes.

Erythrocyte aminotransferase activity is followed by determination of the activity coefficient (AC) for glutamate pyruvate transaminase (alanine aminotransferase, AlAT),

Table 9.1 Symptoms of Vitamin B_6 Deficiency

System	Signs
General	
Appetite	Decrease
Growth	Decrease
Dermatologic	Acrodynia, cheilosis, stomatitis, glossitis
Muscular	Weakness
Skeletal	Dental caries
Organs	Hepatic steatosis
Vascular	
Vessels	Arteriosclerosis
Erythrocytes	Anemia
Nervous	Paralysis, convulsions, peripheral neuropathy
Reproductive	Decrease egg production
Fetal	Malfomations, death
Congenital Disorders	
Homocysteinuria cystathione β-synthetase deficiency	Thrombosis, skeletal and connective tissue malformation, mental retardation
Cystathionuria cystathione γ-lyase deficiency	Mental retardation
γ-Amino butyric acid (GABA) deficiency	Neuropathies
Glutamic decarboxylase deficiency	
Sideroblastic anemia D-aminolevulinate synthase deficiency	Anemia, cystathionuria, xanthuremic aciduria

Source: Combs, G. F., Jr., *The Vitamins: Fundamental Aspects in Nutrition and Health,* Academic Press, New York, 1992, Chap. 13.

which catalyzes the transamination reaction between alanine and α-ketoglutarate to form pyruvate and L-glutamate or aspartate aminotransferase (glutamate oxaloacetate transaminase, AsAT). The AC is calculated after supplementation of the subject with PLP by the following formulas:

$$\text{Erythrocyte AlAT AC or AsAT AC} = \frac{\mu\text{g pyruvate mL}^{-1}\ \text{h}^{-1}\ \text{(with added PLP)}}{\mu\text{g pyruvate mL}^{-1}\ \text{h}^{-1}\ \text{(without added PLP)}}$$

PLP stimulation of the AlAT AC to levels greater than 1.25 can be considered as inadequate status.[4] AsAT AC levels >1.85 indicate vitamin B_6 deficiency.[5]

Since PLP is the coenzyme for kynureninase and kynurenine aminotransferase in the kynurenine stage of the conversion of tryptophan to niacin, decreased activity of these enzymes during vitamin B_6 deficiency leads to increased urinary excretion of xanthurenic acid and kynurenic acid. Excretion levels are increased by oral loading of tryptophan; hence, the status test is known as the tryptophan load test. Plasma PLP levels and the tryptophan load test when concurrently completed provide excellent biochemical confirmation of vitamin B_6 status. Plasma PLP levels correlate with tissue concentrations.[5]

Because of its major role in amino acid metabolism, vitamin B_6 is widely dispersed throughout the plant and animal kingdoms. Data derived from the United States Department of Agriculture (USDA) National Nutrient Database for Standard Reference, Release 19[11] shows that fortified breakfast cereals, fish, poultry, meats, cereal grains, potatoes, fruits, and vegetables are excellent sources of vitamin B_6 (Table 9.3). Breakfast cereals that are supplemented contain ten or more times higher levels compared to the naturally occurring levels.

Table 9.2 PLP-Dependent Enzymes

Reaction	Enzyme
Decarboxylations	Aspartate 1-decarboxylase
	Glutamine decarboxylase
	Ornithine decarboxylase
	Aromatic amino acid decarboxylase
	Histidine decarboxylase
R-group interconversions	Serine hydroxymethyltransferase
	d-Aminolevulinic acid synthase
Transaminations	Aspartate aminotransferase
	Alanine aminotransferase
	γ-Aminobutyrate aminotransferase
	Cysteine aminotransferase
	Tyrosine aminotransferase
	Leucine aminotransferase
	Ornithine aminotransferase
	Glutamine aminotransferase
	Branched-chain amino acid aminotransferase
	Serine-pyruvate amino transferase
	Aromatic amino acid transferase
	Histidine aminotransferase
Racemization	Cystathione β-synthase
α,β-Elimination	Serine dehydratase
γ-Elimination	Cystathione γ-lyase
	Kynureninase

Source: Combs, G. F., Jr., *The Vitamins: Fundamental Aspects in Nutrition and Health*, Academic Press, New York, 1992, Chap. 13.

However, bioavailability, discussed in Section 9.2, is highly variable and poorly documented. Dietary Reference Intake (RDI) values (Table 9.4) for vitamin B_6 provide Recommended Dietary Allowances (RDA) that range from 1 to 1.7 mg d^{-1} for males and 1 to 1.5 mg d^{-1} for females. During pregnancy and lactation, the RDA levels are increased to 1.9 and 2.0 mg d^{-1}, respectively. Tolerable Upper Intake Levels (UL) are set at 100 mg d^{-1} for adult men and women. Adverse effects of high doses that might occur through use of supplements includes sensory neuropathy and dermatological lesions.[6] Median daily intakes of vitamin B_6 are approximately 2 mg d^{-1} for men and 1.5 mg d^{-1} for women.[6] The Reference Daily Intake (RDI) set by the Nutrition Labeling and Education Act of 1990 (NLEA) is 2.0 mg.[12]

9.2 Properties

9.2.1 Chemistry

9.2.1.1 General properties

Vitamin B_6 refers to all 2-methyl-3-hydroxy-5-hydroxy methyl pyridine compounds that possess the biological activity of pyridoxine (PN) (2-methyl-3-hydroxy-4,5-bis (hydroxymethyl)-pyridine) in rats. PN, substituted at the 4-position with a hydroxymethyl group, has been commonly referred to as pyridoxol; however, the preferred name is pyridoxine. Other vitamin B_6 forms distributed throughout nature include pyridoxal (PL) and pyridoxamine (PM) with an aldehyde (–CHO) and aminoethyl (–CH_2NH_2) substituted at the 4-position of the pyridine ring, respectively. The structure and accepted ring numbering system for PN is given in Figure 9.1. Pyridoxal kinase phosphorylates PN, PL, and PM to the 5′-phosphates (PNP, PLP, and PMP). The metabolically active form is PLP. PNP and PMP are

Table 9.3 Vitamin B_6 Content of Various Foods

Description	NDB no.	mg 100 g^{-1}	Description	NDB no.	mg 100 g^{-1}
Cereals ready-to-eat, KELLOGG, KELLOGG'S ALL-BRAN Original	08001	12.00	Turkey, all classes, giblets, cooked, simmered some giblet fat	05172	0.58
Cereals ready-to-eat, GENERAL MILLS, Whole Grain TOTAL	08077	9.41	Nuts, chestnuts, European, roasted	12167	0.50
Cereals ready-to-eat, KELLOGG, KELLOGG'S, Complete Wheat Bran Flakes	08028	7.00	Potatoes, hashed brown, home-prepared	11370	0.47
Cereals ready-to-eat, KELLOGG, KELLOGG'S PRODUCT 19	08058	6.90	Chickpeas (garbanzo beans, Bengal gram), mature seeds, canned	16058	0.47
Cereals ready-to-eat, GENERAL MILLS, TOTAL Corn Flakes	08246	6.67	Turkey, call classes, meat only, cooked, roasted	05168	0.46
Cereals ready-to-eat, KELLOGG, KELLOGG'S SPECIAL K	08067	6.40	Chicken, broilers or fryers, breast, meat and skin, cooked, fried, batter	05058	0.43
Cereals ready-to-eat, QUAKER, CAP'N CRUNCH'S PEANUT BUTTER CRUNCH	08012	4.85	Cereals, CREAM OF WHEAT, mix'n eat, plain, prepared with water	08109	0.40
Cereals ready-to-eat, KELLOGG, KELLOGG'S FROOT LOOPS	08030	3.68	Fish, halibut, Atlantic and Pacific, cooked, dry heat	15037	0.40
Cereals ready-to-eat, GENERAL MILLS, TOTAL Raisin Bran	08247	3.64	Cornmeal, self-rising, degermed, enriched, yellow	20025	0.39
Cereals ready-to-eat, KELLOGG, KELLOGG'S Corn Flakes	08020	3.44	Bananas, raw	09040	0.37
Cereals ready-to-eat, GENERAL MILLS, WHEATIES	08089	3.33	Rice, white, long-grain, parboiled, enriched dry	20046	0.35
Cereals ready-to-eat, GENERAL MILLS, FROSTED WHEATIES	08266	3.33	Fish, haddock, cooked, dry heat	15034	0.35
Cereals ready-to-eat, KELLOGG, KELLOGG'S COCOA KRISPIES	08014	3.28	Bulgur, dry	20012	0.34
Cereals ready-to-eat, QUAKER, QUAKER 100% Natural Cereal with oats, honey, and raisins	08218	2.84	Potatoes, baked, flesh, without salt	11363	0.30
Cereals ready-to-eat, KELLOGG, KELLOGG'S RICE KRISPIES	08065	2.80	Plantains, raw	09277	0.30
Cereals ready-to-eat, GENERAL MILLS, CHEERIOS	08013	2.20	Barley, pearled, raw	20005	0.26
Cereals ready-to-eat, KELLOGG, KELLOGG'S FROSTED FLAKES	08069	1.60	Fast foods, hamburger, large, double patty, with condiments and vegetables	21114	0.24
Snacks, KELLOGG, KELLOGG'S NUTRI-GRAIN Cereal Bars, fruit	19441	1.40	Potatoes, mashed, home-prepared, whole milk added	11657	0.23
Fish, tuna, yellowfin, fresh, cooked, dry heat	15221	1.04	Carrot juice, canned	11655	0.22
Beef, variety meats and by-products, liver, cooked, pan-fried	13327	1.03	Plums, dried (prunes), stewed, without added sugar	09292	0.22
Cereals ready-to-eat, GENERAL MILLS, RAISIN NUT BRAN	08261	0.91	Prune juice, canned	09294	0.22
Cereals ready-to-eat, KELLOGG, KELLOGG'S RAISIN BRAN	08060	0.85	Tomato products, canned, paste without salt added	11546	0.22
Chicken, broilers or fryers, light meat, meat only, cooked, fried	05040	0.63	Sweet potato, canned, vacuum pack	11512	0.19

Source: Data from United States Department of Agriculture, Agricultural Research services, 2006, USDA Nutrient Database for standard Reference, Release 19, Nutrient Data Laboratory Home Page, http://www.nal.usda.gov/fnic/foodcomp, Riverdale, MD, Nutrient Data Laboratory, USDA.

Table 9.4 Dietary Reference Intakes (DRI) and Tolerable Upper Intake Levels (UL) for Vitamin B_6[a]

Life stage	DRI (mg d^{-1})	UL (mg d^{-1})
Infants (months)		
0–6	0.1	[b]
7–12	0.3	[b]
Children (years)		
1–3	**0.5**	30
4–8	**0.6**	40
Males		
9–13	**1.0**	60
14–18	**1.3**	80
19–30	**1.3**	100
31–50	**1.3**	100
51–70	**1.7**	100
>70	**1.7**	100
Females (years)		
9–13	**1.0**	60
14–18	**1.2**	80
19–30	**1.3**	100
31–50	**1.3**	100
51–70	**1.5**	100
>702	**1.5**	100
Pregnancy (years)		
≤18	**1.9**	100
19–30	**1.9**	100
31–50	**1.9**	100
Lactation (years)		
≤18	**2.0**	100
19–30	**2.0**	100
31–50	**2.0**	100

[a] Food and Nutrition Board, Institute of Medicine, *Dietary Reference Intake for Thiamin, Riboflavin, Niacin, Vitamin B_6, Folate, Vitamin B_{12}, Pantothenic Acid, Biotin, and Choline*, National Academy of Sciences Press, Washington, DC, 2000, Chap. 7.

Bold type: Recommended Dietary Allowance; ordinary type: Adequate Intake (AI).

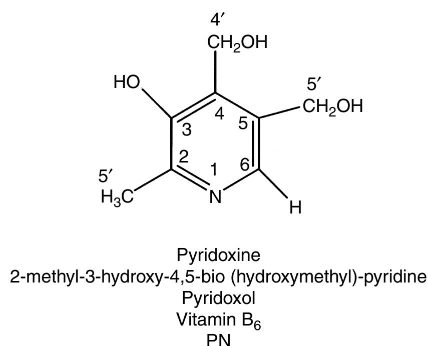

Figure 9.1 Structure of pyridoxine (PN).

oxidized to PLP by PM (PN) 5′-phosphate oxidase.[8] PN, PL, and PM are metabolically interconvertible and considered to be biologically active equivalents. Excellent overviews of the chemical and biochemical properties of vitamin B_6 are provided by Ubbink[13] and Leklem.[14] Since each of the six vitamers is found in food, analytical techniques must be capable of quantification of each, to accurately assess vitamin B_6. Resolution and quantitation of

the phosphorylated forms and 4-PA, the catabolized form excreted in urine, are often required. LC methods, as discussed in Section 9.3, are well suited to these analytical demands. Structures of the six metabolically active vitamers, 4-PA, and pyridoxine-glucoside (PN-glucoside) are given in Figure 9.2.

Physical characteristics of vitamin B_6 are summarized in Table 9.5. Pyridoxine hydrochloride ($C_8H_{12}ClNO_3$, Mwt 205.64) is the commonly available commercial form. The PN·HCl salt is a white, odorless crystalline powder with a slightly salty taste. It presents few problems from a food fortification standpoint. The PN·HCl is readily soluble in water (22 g 100 mL^{-1}), alcohol, and propylene glycol. It is sparingly soluble in acetone and practically insoluble in ethyl ether and chloroform. PN is more stable than PL and PM,

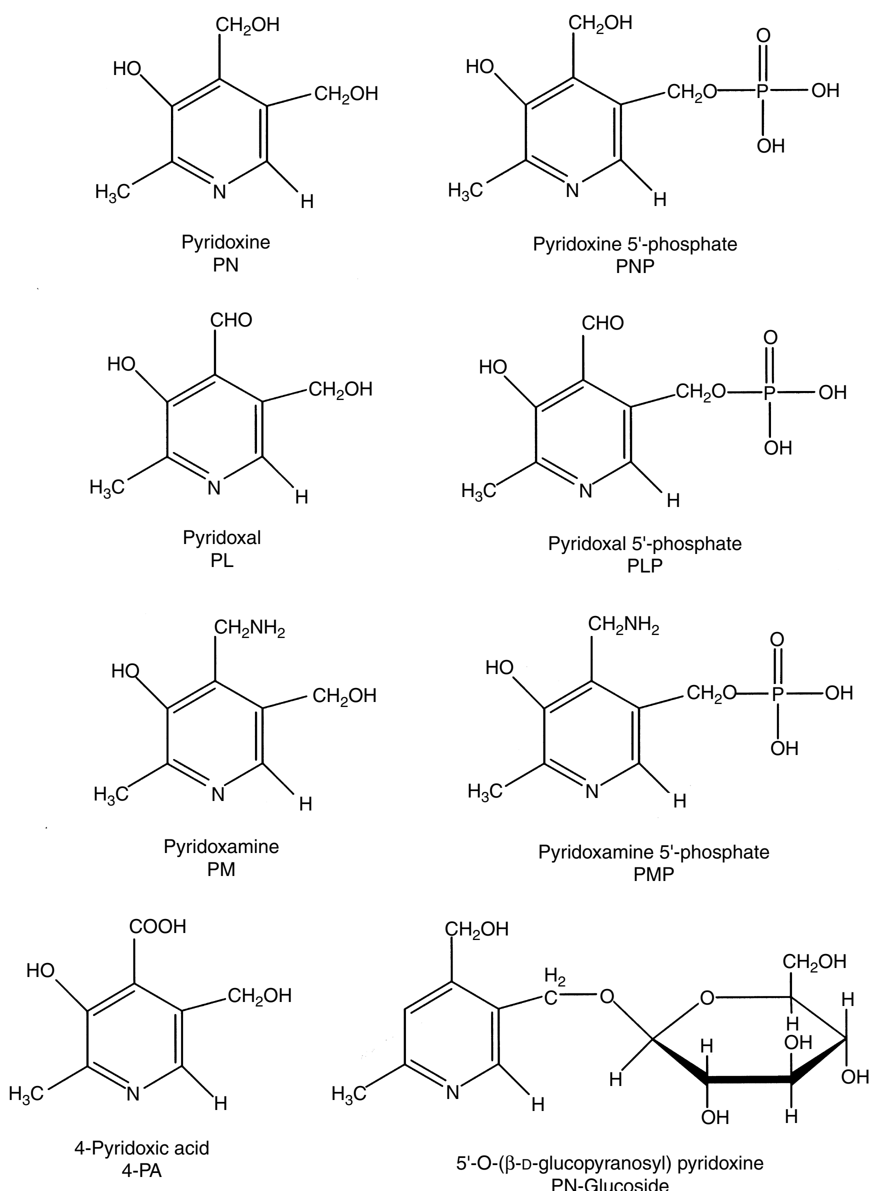

Figure 9.2 Structures of pyridoxine and related compounds.

Table 9.5 Physical Properties of Vitamin B_6

Substance[a]	Molar mass	Formula	Solubility	Melting point °C	Absorbance[b] λ_{max} (nm)	$E^{1\%}_{1cm}$	ε	Solvent	Fluorescence maxima Ex (nm)	Em (nm)	pH range
Pyridoxal HCl	203.63	$C_8H_9NO_3 \cdot$	Soluble in water,	165 (dec.)	390	[9.8]	200	Water	330	382	6.0
CAS No. 65-22-5		HCl	95% ethanol		318	[399]	8128	Water	310	365	12.0
8162											
Pyridoxine HCl	205.64	$C_8H_{12}ClNO_3$	Soluble in water, alcohol,	206–208	292	[375]	7720	Methanol			
CAS No. 58-56-0			propylene glycol,	Sublimes	290	[408]	8400	0.1 N HCl			
8166			sparingly soluble in acetone		253	[180]	3700	Phosphate buffer, pH 7.0			
			Insoluble in ether, $CHCl_3$		325	[345]	7100	Phosphate buffer, pH 7.0			
Pyridoxine	169.18	$C_8H_{11}NO_3$	Soluble in water	160	324	[428]	7244	pH 6.8	332	400	6.5–7.5
CAS No. 65-23-6			Weakly soluble in alcohol, acetone		254	[23]	3891	pH 6.8	320	380	12.0–14.0
8166			Insoluble in ether, $CHCl_3$								
Pyridoxamine Dihydrochloride	241.12	$C_8H_{12}N_2O_2 \cdot$	Soluble in water,	226–227	328	322	7763	Water	337	400	4.0–5.5
CAS No. 524-36-7		2HCl	95% alcohol	(dec.)	253	[190]	4571	Water	320	370	14
8164											
Pyridoxal-5′-phosphate	247.14			330		[101]	2500	Phosphate buffer, pH 7.0	365	423	2.5–4.8
									360	430	8.7–13.0
CAS No. 54-47-7				388		[198]	4900	Phosphate buffer, pH 7.0	330	410	6.0
8163											

[a] Common or generic name; CAS No.: Chemical Abstract Service number, bold print designates the Merck Index monograph number.
[b] Values in brackets are calculated from corresponding $E^{1\%}_{1cm}$ values.

Source: Ubbink, J. B., Vitamin B_6, In *Modern Chromatographic Analysis of Vitamins*, 3rd ed., De Leenheer, A. P., Lambert, W. E., and Van Bocxlaer, J. F., eds., Marcel Dekker, Inc., New York, 2000, chap. 10; Leklem, J. E., Vitamin B_6, In *Handbook of Vitamins*, 3rd ed., Rucker, R. B., Suttie, J. W., McCormick, D. B., and Macklin, L. J., Eds., Marcel Dekker, Inc., New York, 2001, chap. 10; Budavari, S., *The Merck Index*, 13th ed., Merck and Company, Whitehouse Station, NJ, 2001, p. 1428; Friedrich, W., Vitamin B-6, In *Vitamins*, Water de Gruyter, Hawthorne, New York, 1988, chap. 9; Ball, G. F. M., Chemical and biological nature of water-soluble vitamins, In *Water-Soluble Vitamin Assays in Human Nutrition*, Chapman and Hall, New York, 1994, chap. 2; Bridges, J. W., Davies, D. S., and Williams, R. T., Fluorescence studies on some hydroxypyridines including compounds of the vitamin B-6 group, *Biochem. J.*, 98, 451, 1966; Peterson, E. A. and Sober, H. A., Preparation of crystalline phosphorylated derivatives of vitamin B_6, *J. Am. Chem. Soc.*, 76, 169, 1954; Storvick, C. A., Benson, E. M., Edwards, M. A., and Woodring, M. J., Chemical and microbiological determination of vitamin B_6, *Methods Biochem. Anal.*, 12, 183, 1964.

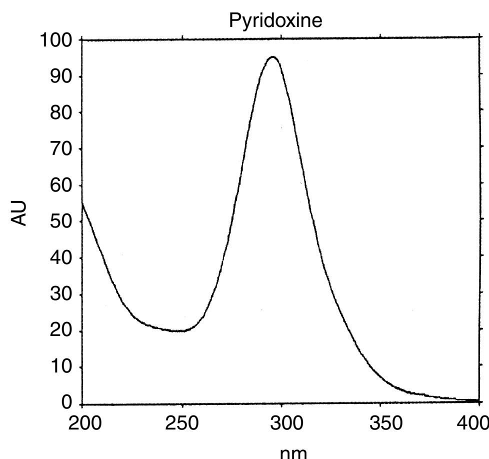

Figure 9.3 UV spectrum of pyridoxine. (Reproduced with permission from Aranda, M. and Morlock, G., *J. Chromatogr. A*, 1131, 253, 2006.)

providing a highly usable form for food fortification and formulation of pharmaceuticals. PN·HCl is the USP reference standard.

9.2.1.2 Spectral properties

Spectral properties of vitamin B_6 compounds and other hydroxypyridines were presented by Bridges et al.,[18] Peterson and Sober,[19] Storvick et al.,[20] Ball,[17] Ubbink,[13] and Leklem[14] recently summarized ultraviolet (UV) and fluorescence properties of the vitamin B_6 group. For specifics of the spectral properties, the reader is referred to the excellent study provided by Bridges et al.[18] PN, PL, PM, and the 5′-phosphates show absorption maxima at 290 nm when dissolved in 0.1 M HCl.[14,18] At pH 7.0, PL and PLP show maximum absorbance at 390 nm; whereas, the other vitamers maximally absorb at 253 and 325 nm.[14,18] The ultraviolet-visible (UV/Vis) spectrum of pyridoxine is shown in Figure 9.3.[137]

Fluorescence properties are given in Table 9.5. Excitation and emission maxima are those originally provided by Bridges et al.[18] The nonphosphorylated vitamers fluoresce strongly from slightly acidic to strongly alkaline pH.[14] PLP fluoresces weakly compared to PMP and PNP, which fluoresce similarly to the respective nonphosphorylated forms. Structurally, PL exists in solution as the hemiacetal that provides for its strong fluorescence intensity. The low-intensity fluorescence of PLP is the result of the phosphate ester on the 5-position of the pyridine ring prohibiting hemiacetal formation.[14,18] Several approaches are available to enhance fluorescence of PLP for effective detection after LC resolution (Section 9.3). Fluorescence provides a highly sensitive and specific detection mode for the analysis of all vitamin B_6 forms commonly encountered in food and other biological samples.

9.2.2 Stability

Model system studies with PN, PL, and PM hydrochloride salts have clearly demonstrated the significance of pH and light to the stability of the different vitamin B_6 forms. Stability characteristics can be summarized as follows:

1. All forms are quite stable in acidic solutions if protected from light.
2. Laboratory light conditions or exposure of foods to light during storage, marketing, or preparation can significantly affect vitamin B_6 retention.[21,22]

3. PN is more stable than PL and PM. PM is the least stable.
4. Light exposure can degrade all forms even at acidic pH values. Stability greatly decreases as pH increases to levels above neutrality.[22]
5. Near-UV and UV radiation are the most damaging wavelengths.[23]
6. Use of low actinic glassware[18] and yellow or golden fluorescent light are essential for the analytical laboratory.[21]
7. Processing and cooking conditions cause variable losses. Heat degradation increases as pH increases. PN is more heat stable than PL and PM.[23,24]
8. Processing losses of vitamin B_6 in vegetables can be large and variable owing to physical loss from leaching. Heat degradation plays a greater role in animal products, since PN is the primary vitamer in plants and PL and PM are more predominant in animal tissues and milk.[25]
9. Interconversion of the vitamin B_6 forms occurs during heat processing of food and during preparation of biological samples. Relative concentrations of the vitamers can dramatically change during processing.[24,25]
10. Pyridoxal can react with the ε-amino group of lysine to form a Schiff base and subsequently rearrange to pyridoxylidenelysine.[26,27]
11. The bound phosphate on PLP can enhance Schiff base formation between the aldehyde group and the ε-amino group. Pyridoxal phosphate degradation is faster than PL in aqueous medium.[26,27]
12. Bioavailability can be significantly affected by food processing (Section 9.2.3).

9.2.3 Bioavailability

Excellent research studies have provided in-depth knowledge on the bioavailability of vitamin B_6. It has been recognized for many years that vitamin B_6 exists in nature in protein and non–protein-bound forms. Extraction procedures designed for biological samples have a primary role to free such bound forms to ensure complete analysis of the vitamin. It is now understood that unique bound forms of vitamin B_6 can affect bioavailability to the animal or human and that interpretation of analytical values for certain foods should be based upon the forms naturally present in the food. A general point is that animal products contain more bioavailable forms of vitamin B_6 compared to plant food sources.

In plants, PN can exist in quite significant amounts as a β-glucoside. The glucoside was identified as 5′-*O*-(β-ᴅ-gluco-pyranosyl) pyridoxine (PN-glucoside, Figure 9.2) as a constituent of rice bran and in germinating seeds of cereals.[28–30] Kabir et al.[31] called attention to the bioavailability question of PN-glucoside for humans by showing an inverse relationship between the glycosylated vitamin B_6 content of food and its bioavailability to humans. Differential assays were developed on the basis of varying extraction procedures (Section 9.3.2.2.1) to differentiate PN and PN-glucoside in foods.[28,32] The first highly descriptive paper on PN-glucoside in common plant foods was provided by Kabir et al.[32] Table 9.6 data clearly show that the levels of PN-glucoside is quite variable in plant products and nonexistent in animal products. In broccoli, cauliflower, carrots, and cooked soybeans greater than 50% of the vitamin B_6 was in the PN-glucoside form. In a later study, PN-glucoside was identified in potatoes and shown to increase during storage.[33] More recently, 5′-O-[6-*O*-((+)-5-hydroxy-dioxindole-3-acetyl)-β-cellobiosyl] pyridoxal was identified in rice bran, representing another confirmed, conjugated form of vitamin B_6.[34]

Studies of PN-glucoside were advanced with the development of an LC procedure for its quantitation.[35] The method, developed by Gregory and Ink,[35] is provided in Section 9.4. Work by Gregory and colleagues[36–38] on the bioavailability of PN-glucoside proved that the substance is poorly available to humans and animals as a source of vitamin B_6. The bioavailability studies conducted by Gregory's group represent some of the most

Table 9.6 Vitamin B_6 and Glycosylated Vitamin B_6 Content of Different Foods

Food	Total vitamin B_6[a] μg 100 g^{-1}	Nonconjugated vitamin B_6[a] μg 100 g^{-1}	%[b]	Glycosylated vitamin B_6[a] μg 100 g^{-1}	%	Sum[c]
Vegetables						
Broccoli, raw	168 ± 0.8[d]	140 ± 4	84	n.d.[e]	—	84
Broccoli, frozen	119 ± 13	48 ± 2	23	78 ± 10	65	88
Cauliflower, raw	156 ± 0.8	148 ± 0.2	95	9 ± 0.5	5	100
Cauliflower, frozen	84 ± 7	20 ± 4	23	69 ± 8	82	105
Green beans, raw	60	51	85	6	10	95
Green beans, canned	28 ± 2	16 ± 0.1	56	8 ± 0.1	28	84
Carrots, raw	170	75	44	87	51	95
Fruits						
Bananas	313 ± 6	308 ± 31	98	10 ± 14	3	101
Avocados, fresh	443 ± 4	221 ± 1	50	15 ± 6	3	53
Orange juice, fresh	43 ± 0.1	18 ± 0.5	42	16 ± 0.6	37	79
Orange juice, concentrate	165 ± 2	54 ± 1	33	78 ± 0.8	47	80
Peaches, canned	9 ± 0.3	7 ± 0.5	71	2 ± 0.1	21	92
Nuts						
Filberts, raw	587 ± 15	707 ± 16	120	26 ± 29	4	124
Almonds, raw	86	69	81	n.d.	0	81
Grains						
Corn, frozen	88 ± 14	38 ± 9	44	6 ± 1	6	50
Rice (white) cooked	138 ± 2	50 ± 2	37	19 ± 1	14	51
Rice bran	3515 ± 84	600 ± 6	17	153 ± 30	4	21
Whole wheat bread	169 ± 2	69 ± 1	40	29 ± 3	17	57
Whole bran	903 ± 1	117 ± 3	13	236 ± 17	36	49
Whole wheat flour	265	129	48	19	11	59
Legumes						
Navy beans, cooked	381 ± 35	143 ± 6	37	159 ± 9	42	79
Peanut butter	302 ± 21	49 ± 5	16	54 ± 11	18	34
Soybeans, cooked	627 ± 11	130 ± 4	21	357 ± 4	57	78
Animal Products						
Beef, ground, cooked	263	83	31	n.d.	—	31
Tuna, canned	316	158	50	n.d.	—	50
Chicken						
Breast, raw	700	454	65	n.d.	—	65
Breast, cooked	684	316	46	n.d.		46
Leg, raw	388	176	45	n.d.		45
Leg, cooked	306	150	49	n.d.		49
Milk, skim	5 ± 1	4 ± 0.3	79	n.d.		79

[a] Total vitamin B_6 refers to the amount of vitamin B_5 measured microbiologically after acid hydrolysis. Nonconjugated vitamin B_6 is the amount measured in a sample that was mixed with 0.1 *M* phosphate buffer for 2 h. Glycosylated vitamin B_6 refers to the difference between the enzyme treated value and the free vitamin B_6 value. All values are as pyridoxine equivalents per 100 g of food.
[b] The percent of nonconjugated and glycosylated forms was calculated by dividing the amount of free or glycosylated forms by the total vitamin B_6 content of each corresponding food.
[c] Sum of the % nonconjugated and glycosylated vitamin B_6 as a percentage of the total vitamin B_6.
[d] The values listed are means ± standard deviation for duplicated samples. Values without a standard deviation are based on a single analysis.
[e] n.d. = not detected by the enzyme treatment.

Source: Reproduced from Kabir, H., Leklem, J., and Miller, L.T., *J. Food Sci.*, 48, 1422, 1983. With permission.

in-depth studies completed on the bioavailability of a water-soluble vitamin form from the food supply.

9.3 Methods

For many reasons, LC analysis is the preferred method for vitamin B_6 assay of biologicals. Food analysis can be accurately completed microbiologically and blood analysis by enzymatic and immunological procedures, but the sensitivity and specificity provided by current LC-based methods make them the clear choice for foods and most other biologicals. Microassay is still in common usage. Excellent spectrophotometric, fluorometric, and electrochemical methods are available, but their use is primarily for analysis of pharmaceuticals. Helpful reviews available on vitamin B_6 assay methods include Polansky et al.,[39] Ubbink,[13] Lumley,[40] Gregory,[41] Ball,[17] and Eitenmiller and Landen.[42] Various handbook and regulatory procedures are summarized in Table 9.7.[43–56]

9.3.1 Microbiological

Several different microorganisms have been used to assay vitamin B_6. None are without problems. Strohecker and Henning[57] in their early text on vitamin assay provided methodology based on *Saccharomyces carlsbergensis* (*S. uvarum*), *Streptococcus faecalis* (*Enterococcus faecalis*), and *Lactobacillus helviticus*. Other organisms used in the past include *Lactobacillus casei*, *Neurospora sitophila*, *Saccharomyces cerevisiae*, and the protozoan, *Tetrahymenia pyriformis*. Of these, the most commonly used and preferred organism is *S. uvarum* (ATCC 9080). The assay was introduced in 1943,[58] and refined for food analysis at the Beltsville Human Nutrition Research Center, United States Department of Agriculture. Studies by Parrish et al.[59,60] showed that *S. uvarum* responded unequally to the three vitamin B_6 vitamers. Response to PN and PL were similar, but growth response to PM was less. The differential growth response to the vitamers led to the development of chromatographic procedures to separate PN, PL, and PM, and then to assay them individually by *S. uvarum*. This procedure forms the basis of AOAC Method 961.15.[45] The chromatographic method as developed by MacArthur and Lehmann,[61] Toepfer and Lehmann,[46] and Toepfer and Polansky[48] is shown in Figure 9.4. Cation-exchange chromatography on Dowex AG50W-X8 resin resolves the vitamers and allows their subsequent individual quantitation.

It is generally accepted that *S. uvarum* gives differential response to PN, PL, and PM. Also, the lower response to PM can be slight or quite significant depending on individual cultures and the laboratory culture procedures in use. Polansky[62] pointed out that when studies are completed with *S. uvarum*, the effects of the differential growth response can be eliminated by use of the chromatographic separation before microbiological assay. The tedious nature of the procedure led to the use of rapid and precise LC procedures capable of resolution of the three vitamers and their phosphate esters. The work by the USDA on the vitamin B_6 assay by *S. uvarum* represents an important era in vitamin B_6 analysis.

Various researchers[63,64] have suggested that *Kloeckera apiculata* (ATCC 9774) replace *S. uvarum* for vitamin B_6 assay. Observations by Barton-Wright[63] and, later, by Guilarte et al.[64] indicated essentially equal growth response by *K. apiculata* to PL, PN, and PM. However, other investigators showed a lower growth response to *K. apiculata* to PM, similar to the growth response of *S. uvarum*, compared to the response to PL and PN.[62,65] *K. apiculata* has not seen wide usage for food analysis. However, Guilarte et al.[66] used *K. apiculata* to develop a radiometric assay for B_6 analysis. Radiometric procedures for vitamin B_6 and other water-soluble vitamin assays have not been widely applied.

Association of Official Analytical Chemists (AOAC) International[45] provides two methods on the basis of microbiological assay with *S. uvarum* (ATCC 9080).

Table 9.7 Regulatory and Handbook Methods for Analysis of Pyridoxine Hydrochloride, Pyridoxine, Pyridoxal, and Pyridoxamine

Source	Form	Methods and application	Approach	Most current cross-reference
***U.S. Pharmacopeia National Formulary*, 2006, USP 29/NF 24, Dietary Supplements Official Monographs**[43]				
1. Pages 2392–2394, 2396, 2399, 2404, 2419–2421	Pyridoxine hydrochloride	Pyridoxine hydrochloride in oil- and water-soluble vitamin capsules/ tablets, oral solution w/wo minerals	Method 1—LC 280 nm Method 2—LC 254 nm Method 3— LC 270 nm	None
2. Pages 2428–2430, 2432–2433, 2435–2436	Pyridoxine hydrochloride	Pyridoxine hydrochloride in water-soluble vitamin capsules/tablets w/wo minerals	Method 1—LC 280 nm Method 2—LC 254 nm Method 3—LC 270 nm	None
3. Pages 1869–1870	Pyridoxine hydrochloride	Pyridoxine hydrochloride (NLT 98.0%, NMT 102.0%)	LC 280 nm	None
4. Pages 1870–1871	Pyridoxine hydrochloride	Pyridoxine hydrochloride injection/tablets	Colormetric 650 nm	None
***British Pharmacopoeia*, 2007**[44]				
1. Pages 1772–1773	Pyridoxine hydrochloride	Pyridoxine hydrochloride	Titration	None
2. Page 2880	Pyridoxine hydrochloride	Pyridoxine tablets	Spectrophotometric 290 nm	None
3. Pages 2989–2990	Pyridoxine hydrochloride	Vitamins B and C injections	LC 280 nm	None
***AOAC Official Methods of Analysis*, 18th ed., 2005**[45]				
1. 45.2.08	Pyridoxine Pyridoxal	AOAC Official Method 961.15, Vitamin B_6 (Pyridoxine, Pyridoxal, Pyridoxamine) in Food Extracts	Microbiological	*J. Assoc. Off. Anal. Chem.*, 44, 426, 1961;[46] *J. Assoc. Off. Anal. Chem.*, 47, 750, 1964;[47] *J. Assoc. Off. Anal. Chem.*, 53, 546, 1970[48]
2. 50.1.18	Pyridoxine Pyridoxal Pyridoxamine	AOAC Official Method 985.32, Vitamin B_6 (Pyridoxine, Pyridoxal, Pyridoxamine) in Ready-To-Feed Milk Based Infant Formula	Microbiological	*J. Assoc. Off. Anal. Chem.*, 68, 514, 1985[49]
3. 50.1.26	Total Vitamin B_6	AOAC Official Method 2004.07, Vitamin B_6 in Reconstituted Infant Formula	LC Fluorescence Ex $\lambda = 290$ Em $\lambda = 395$	*J. AOAC Int.*, 84, 1593, 2001;[50] *J. AOAC Int.*, 88, 30, 2005;[51] *Food Chem.*, 48, 321, 1943;[52] *Food Chem.*, 52, 81, 1995[53]
American Association of Cereal Chemists, *Approved Methods*, vol. 2, 2000[54]				
1. AACC 86–31	Pyridoxine Pyridoxal Pyridoxamine	Vitamin B_6 complete in cereal products	Microbiological	*Official Methods of Analysis, AOAC Int.*, 18th ed.[45]
***Food Chemicals Codex*, 5th ed., 2004**[55]				
1. page 334	Pyridoxine Hydrochloride	Pyridoxine hydrochloride (NLT 98.0%, NMT 100.5%)	Titration, perchloric acid	None

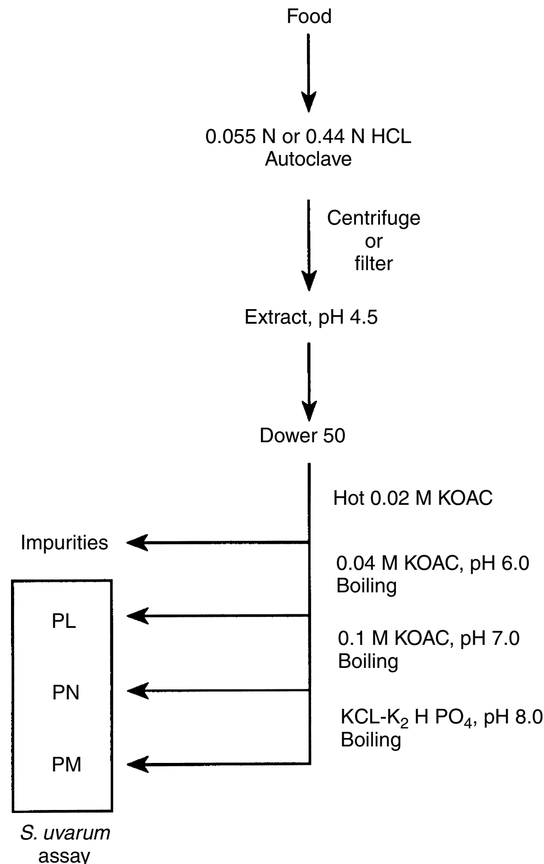

Figure 9.4 Open-column fractionation of PN, PL, and PM for assay by *S. uvarum*. AOAC Method 961.15.

AOAC Official Method 961.15, Vitamin B_6 (Pyridoxine, Pyridoxal, Pyridoxamine) in Food Extracts, Microbiological Method, AOAC Official Methods of Analysis, 45.2.08

Method 961.15 and most other applications of *S. uvarum* rely on early studies[67,68] that established acid extraction procedures suitable for efficient extraction of vitamin B_6 from most food matrices. It was shown that optimal extraction of vitamin B_6 from plant tissues can be achieved by autoclaving the tissue in 0.44 N HCl for 2 h at 121°C. Animal products and tissues are hydrolyzed with 0.055 N HCl by autoclaving at 121°C for 5 h. These extraction procedures have not been modified to any great extent over the years. Following digestion, the hydrolyzates are cooled, adjusted to pH 4.5 with 6 N KOH, and diluted with water. The digests are filtered and chromatographed on Dowex AG 50W-X8 according to procedures established by Toepfer and Lehmann[46] and Toepfer and Polansky.[48] The procedure is tedious, cumbersome, and generally disliked by nutritional analysts. Hot 0.02 M potassium acetate, pH 5.5 is used to elute impurities. Boiling 0.04 M potassium acetate, pH 6.0, is used to elute PL, followed by boiling 0.1 M potassium acetate, pH 7.0, for PN and boiling KCl-K_2HPO_4, pH 8.0, for elution of PM. Each vitamer is individually quantitated and summed to obtain total vitamin B_6 (Figure 9.4). Polansky[62] detailed the historical development and application of the procedure.

AOAC Official Method 985.32, Vitamin B_6 (Pyridoxine, Pyridoxal, Pyridoxamine), in Ready-To-Feed Milk-Based Infant Formula, Microbiological Method, *AOAC Official Methods of Analysis*, 50.1.18

Method 985.32 has been approved only for milk-based infant formula. The method does not require the chromatography step since the primary form of vitamin B_6 is PN added during manufacture of the formula. Vitamin B_6 is extracted with 0.05 M HCl and autoclaved for 5 h. The procedure was provided as a collaborative study in 1985.[49]

9.3.2 *Advances in the analysis of vitamin B_6*

9.3.2.1 *Spectroscopic, electrochemical, and capillary electrophoresis methods*

Excellent spectroscopic methods using spectrophotometric or fluorometric measurements are available for assay of vitamin B_6. Usually, these procedures are applicable to pharmaceuticals. Liquid chromatography procedures are normally required for biologicals and foods owing to greater sensitivity requirements, problems introduced by matrix complexity, and the need to measure PN, PL, and PM as well as the phosphate esters. Spectrophotometric procedures utilize measurement of UV absorbance of the analyte, measurement of colored complexes usually formed through diazotization and coupling reactions or by various derivative spectroscopic methods, which are useful when multiple analytes are present with spectral overlapping. Derivative spectrophotometry often provides increased sensitivity and selectivity compared to nonderivative spectrophotometric methods.[69,74–76,83]

Spectrofluorometric methods, while not as commonly used as spectrophotometric procedures, offer excellent sensitivity and selectivity. Derivative spectrofluorimetry can be used for quantitation of vitamin B_6 combined with analytes with emission spectra close to the emission spectra of vitamin B_6. Flow injection or sequential injection analysis has been used to automate spectrophotometric and spectrofluorometric procedures for analysis of vitamin B_6 in pharmaceuticals.[72,73,78,79,86]

Electrochemical methods are becoming more common for analysis of vitamin B_6.[86–92] Early assays followed the oxidation of PN at a carbon paste electrode. Flow injection and sequential injection techniques and availability of potentiometric membrane sensors and modified glassy carbon electrodes have added speed and improved selectivity to electrochemical assays for vitamin B_6.

Capillary zone electrophoresis (CZE) is often used to quantitate water-soluble vitamin mixtures containing vitamin B_6. Multianalyte methods are discussed in Chapter 14. Klampfl and Vo[70] compared different CZE methods with acidic and basic carrier electrolytes combined with several detection methods for determination of vitamin B_6 and amino acids in infusion solutions. Either UV or mass spectrometric detection with CZE conditions of a 50 mM phosphoric acid, 35 mM octane sulfonate buffer, pH 2.2, adjusted with LiOH, provided conditions for resolution of all analytes, including vitamin B_6. Summaries of spectroscopic, electrochemical, and capillary electrophoresis methods for the analysis of vitamin B_6 are given in Table 9.8.[69–95]

9.3.2.2 *Liquid chromatography*

Problems associated with the microbiological assay of vitamin B_6 led to the early application of LC procedures for food and other biological matrices. Ubbink[13] and Gregory[41] provide in-depth reviews of LC procedures for vitamin B_6 analysis. Ubbink[13] classified applications of LC methods for vitamin B_6 analysis into the following categories:

1. For nutrition survey studies, sensitive methods for determination of plasma/whole blood PLP have been developed. Many of these methods include PL, and sometimes even 4-PA.
2. Specific methods have been described to determine urinary 4-PA excretion, which is frequently used as index of vitamin B_6 nutritional status.

Table 9.8 Selected Spectroscopic, Electrochemical, and Capillary Electrophoresis Methods for the Analysis of Vitamin B_6

Matrix	Description	Accuracy/precision	References
Spectrophotometric			
Pharmaceuticals PN, PM, PL	Vitamins were coupled with the diazonium ion of *P*-sulfanilic acid in a micellar medium of *N*-cetylpyridium chloride, 450 nm	DL (m) PN—3×10^{-7} PM—4×10^{-7} PL—2×10^{-7}	*Anal. Lett.*, 32, 51, 1999[71]
Pharmaceuticals PN	FIA, continuous—flow solid phase sensing Vitamin B_6 is transiently retained on Sephadex SP C-25 cation exchange gel beads in the detection area of the flow cell, 290 nm, throughput = 32–44 h^{-1}	DL—0.02–0.08μ g mL^{-1} QL—0.26–0.07μ g mL^{-1}	*Int. J. Pharmaceut.*, 202, 113, 2000[72]
Pharmaceuticals PN	FIA PN was coupled to the diazonium ion of P-sulfanilic acid in a micellar medium of *N*-cetylpridium chloride, 450 nm, throughput = 60 h^{-1}	DL—5.0×10^{-5} M	*Anal. Lett.*, 33, 539, 2000[73]
Pharmaceuticals PN, thiamin	Ratio Spectra Derivative Spectrophotometry and Matrix Mass Resolution The derivative of the ratio spectra of PN was made at 297.8 and 309.5 nm. For the mass resolution method A1 1 (1%, 1 cm) values were determined at 246.8 and 290.5 nm in the zero-order spectra. Matlab software was used to solve the matric to calculate concentration.	%Recovery matrix resolution 98.3–102.1 Ratio Spectra 98.5–101.3	*J. Pharmaceut. Biomed. Anal.*, 22, 915, 2000[74]
Pharmaceuticals PN	First derivative spectrophotometry Spectral interferences was cancelled out at the zero-crossing wavelength at 306 nm for oral solutions and at 308 nm for injectables and capsules	%Recovery—97.2–100.3 %CV—0.28–0.76	*Anal. Lett.*, 34, 1875, 2001[75]
Pharmaceuticals PN, isoniazid	Derivative Ratio and Differential Derivative Spectrophotometry First derivative ratio spectra of PN and isonazide were determined at 250.7 and 305.8 nm, respectively. ΔD_1 values (signals at zero-crossings of izoniazide and PN) were used to simultaneously determine drug concentration	%Recovery ratio derivative 99 Differential derivative 99	*Spectrosc. Lett.*, 34, 745, 2001[69]
Pharmaceuticals PN, melatonin	First derivative spectrophotometry First derivative amplitudes at 218.3 and 232.7 nm for melatonin and 212.3, 282.6, and 304.6 nm for PN were selected	DL (μg mL^{-1}) PN 0.20 for 212.3 nm 0.12 for 282.6 nm 0.28 for 304 nm	*Anal. Lett.*, 35, 2305, 2002[76]

Continued

Table 9.8 (Continued)

Matrix	Description	Accuracy/precision	References
Spectrophotometric (*Continued*)			
Pharmaceuticals PN plus metoclopramide PN plus meclozine	Binary mixtures were resolved using partial least squares and principle component regression of zero and first order UV spectra of the binary mixtures	DL—8.92×10^{-3} μg mL^{-1} QL—29.7×10^{-3} μg mL^{-1}	*J. Pharmaceut. Biomed. Anal.*, 32, 277, 2003[77]
Pharmaceuticals PN	FIA, Flow injection—solid phase spectrophotometry PN in 0.1 M phosphate buffer, pH 7.5, was injected into a carrier stream of methanol and the phosphate buffer, pH 7.0 (1:1). Blue indophenol dye produced from the reaction of PN with *N*,*N*-diethyl-*p*-pheylenediamine after oxidation by potassium hexacyanoferrate(III) was retained on C_{18} support with detection at 633 nm, throughput = 15 h^{-1}	DL (235 mL)— 0.15 mg L^{-1}	*J. Pharmaceut. Biomed. Anal.*, 34, 543, 2004[78]
Fluorometric			
Pharmaceuticals PN	FIA PN was retained on Sephadex SP-C25 resin placed in the flow cell of a fluorescence detector, Ex λ = 295, Em λ = 385, throughput = 40 h^{-1} at 200 μL injection volume	DL (200 μL)— 5.7 ng mL^{-1}	*Fresenius J. Anal. Chem.*, 363, 265, 1999[79]
Pharmaceuticals PN, codeine, acetylsalicylic acid	The analytes with overlapping emission spectra were simultaneously analyzed spectrofluorometrically by partial least-squares multivariate calibration	—	*J. Pharmaceut. Biomed. Anal.*, 23, 837, 2000[80]
Pharmaceuticals PN, riboflavin, thiamin	Synchronous fluorescence spectrometry of the vitamins in the presence of micellar bis-2-ethylhexy-sulfosuccinate sodium salt was used for quantitation	DL (μg L^{-1}) PN—10 B_1—12 B_2—9 %RSD—1.1–1.3	*Anal. Chim. Acta*, 434, 193, 2001[81]
Tissues PL	PL was dehydrogenated by pyridoxal dehydrogenase to 4-pyridoxolactone. Fluorescence intensity at Ex λ = 356, Em λ = 432 was measured. The assay permitted assay of PL in the presence of PLP	—	*Biosci. Biotechnol. Biochem.*, 66, 1152, 2002[82]
PN, codeine	First derivative spectrofluorometry Emission spectra (275–475 nm) at Ex λ 255 were used to simultaneously assay PN and codeine	DL (μg mL^{-1}) PN—0.02 Codeine—0.11 QL (μg mL^{-1}) PN—0.06 Codeine—0.35	*J. AOAC Int.*, 85, 861, 2002[83]

PN, pyroxicam	Pyroxicam is extracted from the sample matrix with solid phase extraction with C_{18} membranes (C_{18} AR). PN is then assayed fluorometrically in the extract. Piroxicam is assayed fluorometrically on the C_{18} AR membrane	DL (μg mL^{-1}) PN—0.18 Piroxicam—0.01 QL (μg mL^{-1}) PN—0.52 Piroxicam—0.032	*Anal. Chim. Acta*, 466, 275, 2002[84]
Pharmaceuticals PN	PN was absorbed on Sephadex SP C-25 beads packed iral-mm quartz cell, Ex λ = 295, Em λ = 395	DL—0.6–9.5 μg L^{-1} for sample volumes of 10–50 mL	*Spectrosc. Lett.*, 36, 133, 2003[85]
Electrochemical			
Pharmaceuticals PN	SIA (Sequential Injection Analysis) A potentiometric assay with a tubular vitamin B_6 selective flow-through electrode versus AgCl/Ag reference electrode	%Recovery—97.4–103.5	*Pharmaceut. Biomed. Anal.*, 25, 713, 2001[86]
Pharmaceuticals PN, melatonin	Cyclic and Linear Scan Voltametry PN and melatonin were simultaneously assayed by differential pulse voltammetry through oxidation at a glassey carbon working electrode with a Ag/AgCl reference electrode	DL (M) PN—2.45×10^{-6} Melatonin—5.86×10^{-6}	*Pharmazie*, 56, 12, 2001[87]
Pharmaceuticals PN	Potentiometric selective membrane sensors were developed for PN. Sensors were ion-association compleces of PN cation with molybdophosphate and tungstophosphate counter ions as ion pairs in a plasticized PVC matrix	DL—4×10^{-5} M %Recovery—98–99	*Ann. Chim.*, 93, 691, 2003;[88] *J. Anal. Chem.*, 58, 1073, 2003[89]
Pharmaceuticals PN	CME A copper(II) hexacyanoferrate(III) CME was used for PN analysis by cyclic voltammetry, CME (working electrode), SCE (reference electrode), platinum wire (counter electrode)	DL—4.1×10^{-7} mol L^{-1}	*J. Braz. Chem. Soc.*, 14, 316, 2003[90]
Pharmaceuticals PN	CME A carbon paste electrode modified with *N*,*N*-ethylene-bis(salicylideneiminato) oxovanadium(IV) complex was used to assay PN with cyclic voltammetry	DL—3.7×10^{-5} mol L^{-1}	*Anal. Chim. Acta*, 508, 79, 2004[91]
Pharmaceuticals PN	CME A multiwall carbon nanotube modified glassy carbon electrode enhanced the oxidation peak current of PN, modified glassey carbon (working electrode), SCE (reference electrode), platinum (counter electrode)	DL—2×10^{-7} mol L^{-1}	*J. Pharmaceut. Biomed. Anal.*, 36, 631, 2004[92]

Continued

Table 9.8 (Continued)

Matrix	Description	Accuracy/precision	References
Capillary Electrophoresis			
PN, PL, PM, PMP, PLP, pyridoxic acid	MECC 1 meter × 75 μm, 0.01 M phosphate, 0.006 M borate, 0.05 M SDS, 30 kV, fluorescence (Ex λ = 325, Em λ = 430)	LD (pg) PN—750 PM—500 PL—150 PMP—180 PLP—900 Pyridoxic acid—800 fg	*J. Chromatogr.* Sci., 24, 347, 1986[93]
Infusion solutions PN, amino acids	CZE 70 cm × 50 μm, 50 mM phosphoric acid, 25 mM octane sulfonic acid, adjusted to pH 2.2 with LiOH, 25 kV, 195 nm or mass spectrometry	DL—3.0 mg L^{-1}	*J. Liq. Chromatogr. Rel. Technol.*, 26, 2783, 2003[70]
Soft drinks, supplements PN	MECC 50 cm × 75 μm, 50 mM borate, pH 8.5, 25 mM SDS, 30 kV, 214 nm	DL—0.07 μg mL^{-1} QL—0.2 μg mL^{-1}	*Nahrung/Food*, 47, 243, 2003[94]
Serum PN, PL, PM, PLP	CZE 40 cm 75 μm, 30 mM KH_2PO_4, adjusted to pH 8.5 with formic acid, 25 kV, PDA or fluorescence	PN (fluorescence) $\%RSD_r$—3.64 $\%RSD_R$—6.78	*Electrophoresis*, 26, 2376, 2005[95]

3. High performance liquid chromatography (HPLC) methods designed to separate and quantify the six major forms of vitamin B_6 and 4-PA in biological material, are termed comprehensive methods in this review. These methods were usually applied in studies of human vitamin B_6 metabolism or pharmacokinetics of pyridoxine supplementation.
4. Food analysis also requires comprehensive vitamin B_6 analysis, although differentiation between unphosphorylated vitamers and the 5′ phosphoric acid esters is usually not required. Recent research has shown that PN-glucoside has a much lower bioavailability than pyridoxine,[8,62,63] and it is, therefore, sensible to assay PN-glucoside separately.
5. Analysis of pharmaceutical preparations is relatively simple when compared to analysis of biological material, because only PN is used as supplementary B_6 vitamer. Methods for PN determination are usually optimized to include determination of other vitamins of the B group.

We have summarized in Table 9.9 historically important method development publications and selected more recent methods of the application of LC to vitamin B_6 analysis.[50–53,96–137] The most notable advantage of LC analysis compared with microbiological analysis is the high degree of specificity provided, allowing the quantitation of the six vitamers and various metabolites, such as 4-PA. Further, utilization of mass spectrometry combined with resolution capabilities of LC offers added selectivity to vitamin B_6 assays.

9.3.2.2.1 Extraction procedures for analysis of vitamin B_6 by LC. Extraction methods used before LC analysis can be categorized into the following approaches:

Approach 1: Hydrolysis of the phosphate esters with subsequent quantitation of PL, PM, and PN. Hydrolysis is usually enzymatically completed with a commercial phosphatase. Chromatography is simplified, but complete enzymatic conversion must be assured.[41] Treatment with H_2SO_4 has been used.[56]

Approach 2: Preservation of the phosphorylated vitamins with quantitation of the six biologically active forms, glycosylated forms (PN-glucoside), and metabolites such as 4-PA. Extraction is completed with deproteinizing agents such as sulfosalicylic acid (SSA), trichloroacetic acid (TCA), metaphosphoric acid (MPA), and perchloric acid. Sample extraction at room temperature hydrolyzes Schiff bases, prevents enzymatic interconversions of the vitamers and increases water solubility by conversion of the pyridine bases to quaternary ammonium salts.[17] A major advantage is the ability to avoid hydrolysis of PN-glucoside. Acid hydrolysis procedures necessary for microbiological analysis hydrolyze PN-glucoside, therefore, biologically available vitamin B_6 is overestimated.

Approach 3: Conversion of all forms to PN, with quantitation of total vitamin B_6 as PN.[52]

Approach 4: Extraction of all nonbound vitamin B_6 with quantitation as free vitamin B_6. Such extractions can be used to determine relative amounts of free and bound vitamin B_6. Values obtained with required mild extractants such as 0.01 M sodium acetate[33] significantly underestimate nutritionally available vitamin B_6.

Ndaw et al.[138] studied combined enzyme treatments to liberate thiamin, riboflavin, and vitamin B_6 from foods in a single digestion. The work showed that a combination of α-amylase, papain, and acid phosphatase liberated the vitamins and completed dephosphorylation. Use of the protease eliminated the need for acid hydrolysis. The method gave values for reference materials (CRM 487, pig's liver and CRM 421, milk powder) within the certified ranges for each vitamin. When glycosylated forms of vitamin B_6 were noted, addition of β-glucosidase to the enzyme mixture liberated the vitamin B_6. The Ndaw et al.[138]

Table 9.9 LC and LC-MS Methods for the Analysis of Vitamin B_6 in Foods, Feed, Pharmaceuticals, and Biologicals

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods					
Plasma, various foods/PL, PM, PN, PLP, PMP, PNP, 4-PA	Add 3-hydroxypyridine (IS) to 1g sample. Homogenize w/10 mL 5% SSA, centrifuge, 4°C, filter. Evaporate under N_2 to 0.75 original volume. Add equal volume Hex, vortex, draw off lower water layer, and filter. Clean-up: SSA removed by ion-exchange	Column 1—Aminex A-25, 55°C, 24 cm × 6 mm Column 2—Aminex A-25, 18°C, 24 cm × 3 mm. Column in series connected by a switching valve Mobile phase pH gradient—0.4 M NaCl: 0.01 glycine Flow rate—1.2 mL min^{-1} Isocratic—0.4 M NaCl, 0.01 M glycine, 0.05 M semi-carbazine, pH 10 or gradient	Fluorescence PM, PMP, PN, PNP Ex λ = 310 Em λ = 380 PL, PLP Ex λ = 280 Em λ = 487	%Recovery—95–105 %RSD—3	*J. Chromatogr.*, 176, 280, 1979;[96] 196, 176, 1980;[97] 216, 338, 1981;[98] *J. Agric. Food Chem.*, 28, 1145, 1980;[99] *J. Food Sci.*, 46, 943, 1981;[100] *Am. J. Clin. Nutr.*, 37, 867, 1983[101]
Human milk/ PL, PM, PN	Add 4′-deoxypyridine (IS). Digest w/potato acid phosphatase, 1h, 37°C. Add TCA, $CHCl_3$, centrifuge. Adjust supernatant to pH 5.2, filter.	μBondapak C_{18}, 10 μm, 30 cm × 3.9 mm Mobile phase—gradient A—MeOH:water (85:15) B—0.005 M heptane sulfonic acid in 1% HAC.	Fluorescence Ex λ = 300 Em λ = 375	DL (on column) < 1 ng for PM, PN %Recovery PL—105 PN—83 QL—6 μg L^{-1}	*J. Chromatogr.*, 337, 249, 1985;[102] *J. Am. Diet. Assoc.*, 102, 1433, 2002[103]
Chicken, raw, cooked/PL, PM, PLP, PMP	Extract w/1 M MPA and water, Polytron®, filter.	Biosil ODS-55, 25 cm × 4 mm Mobile phase—isocratic 0.066 M KH_2PO_4, pH 3.0	Fluorescence Ex λ = 290 Em λ = 395	DL (on column)—0.1–1.0 ng %Recovery—90–108	*J. Food Sci.*, 53, 371, 1988[104]
Various foods/ PL, PM, PN, PLP, PMP, PNP, 4-PA	Add 4-deoxypyridoxine (IS). Homogenize w/0.1–0.5 M cold perchloric acid, centrifuge. Adjust supernatant to pH 7.5 and after filtration to pH 4.0. Digest w/pH 7.5 filtrate w/alkaline phosphate, 30 min, 25°C. Adjust to pH 4.0	Lichrosphere RP-18, 5 μm, 12.5 cm × 4 mm Mobile phase—gradient A—MeOH B—0.03 M KH_2PO_4, pH 2.7 Flow rate—1.5 mL min^{-1}	Fluorescence Post-column derivatization sodium bisulfate Ex λ = 300 Em λ = 375	DL (on column)—0.4–0.7 pmol %CV—1.1–5.0	*J. Chromatogr.*, 463, 207, 1989[105]

Various foods/ conversion of vitamins to PN	Add 25 mL 0.05 NaOAC, pH 4.5, 25 mL 1 ml glyoxylic acid, pH 4.5, 400 μL ferrous sulfate (2 g L^{-1}) and 20 mg acid phosphatase to 2.5 g sample. Incubate overnight, 37°C, dilute w/water, filter. To 5 mL aliquot, add 4.5 mL 0.1 M sodium borohydride, shake, add 0.5 mL glacial HAC, filter	Lichrosorb 60, RP select B, 5 μm, 25 cm × 5 mm Mobile phase—isocratic MeCN:0.05 M KH_2PO_4 (4:96) containing 0.5×10^{-3} M Na heptane sulfonate, pH 2.5 Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 395	%RSD—3–18 %Recovery—90 QL—0.02 μg g^{-1} %CV < 8	*Food Chem.*, 48, 321, 1993;[52] 52, 81, 1995;[53] *J. AOAC Int.*, 84, 1593, 2001;[50] 88, 30, 2005[51]
Wheat/PL, PM, PN, PMP, PN-glucoside	Add 4′-deoxypyridoxine (IS). Homogenize in water. Deproteinize w/MPA. Centrifuge, filter	Ultramex C_{18}, 5 μm, 15 cm × 4.6 mm Mobile phase—gradient A—0.033 M KH_2PO_4:0.008 M 1-octane sulfonic acid, pH 2.2 B—0.033 M phosphoric acid: 10% MeCN, pH 2.2 Flow rate—1.2 mL min^{-1}	Fluorescence Ex λ = 311 Em λ = 360	%Recovery PN—101 PM—96 PL—57 PLP—34 PMP—79	*Cereal Chem.*, 72, 217, 1995[106]
Pork meat and products/ PM, PL, PN	Add 60 mL 0.1 M HCl and 3 mL 100 μg mL^{-1} 4-deoxypyridoxine (IS) to 5 g sample, shake, stand in water bath at 100°C, 30 min. Cool, adjust to pH 4–4.5. Add 5 mL 10% takadiastase, incubate. Add 2 mL 50% TCA, heat, 95–100°C, 5 min. Dilute to 100 mL w/water, filter	Spherisorb ODS C_{18}, 5 μm, 25 cm × 4 mm Mobile phase—isocratic M H_2SO_4 Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 395	%RSD—6.9-7.0 %Recovery—85–99	*J. Chromatogr.*, 795, 383, 1998[107]
Cooked sausages/PN, PL, PM, PLP, PMP	20 mL 5% metaphosphoric acid, homogenize, dilute to 100 mL w/water. Centrifuge, filter upper solution	Hypersil ODS C_{18}, 5 μm, 10 cm × 4.6 mm Mobile phase—50 mM phosphate buffer (pH 3.2): MeCN (99:1)	Fluorescence Ex λ = 290 Em λ = 395	DL—0.017–0.5 mg $100g^{-1}$ $\%RSD_{intra}$—0.5 $\%RSD_{inter}$—4.3 %Recovery—92–100	*J. Agric. Food Chem.*, 49, 38, 2001[108]

Continued

Table 9.9 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods (*Continued*)					
Various foods/ PL, PM, PN	Add 50 mL 0.1 M HCl to sample, autoclave, 121°C, 30 min. Cool, adjust to pH 4.5, dilute to 100 mL w/water. Shake, centrifuge, filter. Add 1 mL 25 Unit mL^{-1} acid phosphatase to 15 mL filtrate, incubate, 45°C, 18h. Add 5 mL 1 M HCl, dilute to 30 mL w/0.01 M HCl. Filter	Hypersil C_{18}, 3 μm, 15 cm × 4.6 mm Mobile phase—isocratic Buffer mixture (pH 2.75): MeCN (93:7) Flow rate—1 mL min^{-1} Adjust mobile phase through post column infusion to pH 7.5 to improve the detector specificity.	Fluorescence Ex λ = 333 Em λ = 375	DL—5 μg $100g^{-1}$ %RSD—2.6–6.3 %Recovery—96	*Food Chem.*, 82, 315, 2003[109]
Various foods/ PMP, PM, PLP, PL, PN, 4-PA	Acid hydrolysis—add 4 mL 1 M perchloric acid, homogenize. Centrifuge, adjust supernatant to pH 7, stand in ice bath, 30 min. Centrifuge, dilute supernatant to 5 mL w/mobile phase, and filter Enzymatic hydrolysis—adjust pH to 4-4.5 after acid hydrolysis, add 100 mg takadiastase 20 mg $phosphatase^{-1}$ mixture, stir, 50°C, 1h. Adjust supernatant to pH 7, centrifuge, dilute to 5 mL w/mobile phase, and filter	Discovery RP-Amide C_{16}, 5 μm, 15 cm × 4.6 mm Mobile phase—isocratic 50 mM KH_2PO_4, pH 7 Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 335 Em λ = 389	DL—1-27 ng mL^{-1} QL—4-91 ng mL^{-1} %Recovery—94 %RSD—2.6–5.8	*Chromatographia*, 59, 381, 2004[110]
HPTLC-MS					
Energy drinks/ PN, riboflavin, nicotinamide, caffeine, taurine	Degas, apply to HPTLC plates, elute compounds from the plate online into the MS	—	MS-ESI Positive ion mode	%RSD—0.5–4.0	*J. Chromatogr.* A, 1131, 253, 2006[137]

Biologicals					
Tissue/PLP	Homogenize in cold 0.1 M KH_2PO_4, pH 7.0. Deproteinize w/1 N perchloric acid, adjust pH to 6–7 w/3 N KOH. Allow $KClO_4$ to precipitate	LiChrosorb RP-8, 10 μm, 25 cm × 4.6 mm Mobile phase—isocratic 0.033 M KH_2PO_4 (pH 2.5): MeCN (25:75) Flow rate—1.3 mL min^{-1}	Precolumn derivatization Fluorescence Ex λ = 365 Em λ = 400	%Recovery—66–106 QL—0.2 μg mL^{-1}	*Anal. Biochem.*, 102, 374, 1980[111]
Plasma/PL, PM, PN, PLP, PMP, PNP, 4-PA	Transfer aliquot of supernatant. Covert PLP to PLP-semi-carbozone w/semi-carbazide HCl 3-hydroxyproline—IS	Bio-Rad A-25, column-switching, 24 cm × 6 mm, 50°C 24 cm × 3 mm, 18°C Mobile phase—isocratic 0.4 M NaOH:0.01 M glycine: 0.005 M semi-carbazide, pH 10 Flow rate—1.25 mL min^{-1}	Fluorescence PLP Ex λ = 280 Em λ = 487 Others Ex λ = 310 Em λ = 380	QL < 1 ng mL^{-1} %CV—5 %Recovery—98	*J. Chromatogr.*, 196, 176, 1980[112]
Blood/PLP	Add 5 mL 6% TCA to 1 mL blood, vortex. Allow to stand 1h. Mix and centrifuge	Hypersil-ODS, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic 0.05 mM KH_2PO_4, pH 2.9 Flow rate—0.9 mL min^{-1}	Post-column Fluoresence Ex λ = 367 Em λ = 478	QL—5 mmol L^{-1} %CV—3.3–7.1 %Recovery—99–100	Int. J. Vit. *Nutr. Res.*, 51, 216, 1981[113]
Tissue/PL, PM, PN, PLP, PMP, PNP, 4-PA	Add 3-hydroxyproline (IS), extract w/perchloric acid. Adjust pH to 4.2 w/3 M KOH, stand overnight, centrifuge. Clean-up: Dowex H^+, if required	μBondapak C_{18}, 30 cm × 3.9 mm Mobile phase—gradient A—10% IPA containing .09% HAC B—a containing 0.004 M sodium heptane sulfonate: 0.004 M sodium-1-octane sulfonate.	313 nm	—	*J. Chromatogr.*, 227, 181, 1982[114]
Plasma, tissue, food/PL, PM, PN, PMP, PNP, 4-PA, other metabolites	Add TCA, mix, centrifuge. Extract supernatant w/EtO_2. Add 2-amino-5-chlorobenzoic acid (IS) prior to injection	Vydac 401 TP-B, 10 μm, 30 cm × 4.6 mm Mobile phase—gradient A—0.02 N HCl B—0.1 M NaH_2PO_4, pH 3.3 C—0.05 M NaH_2PO_4, pH 5.9	Fluorescence Ex λ = 330 Em λ = 400	—	*Anal. Biochem.*, 129, 310, 1983;[115] *Meth. Enzymol.*, 280, 22, 1997[116]

Continued

Table 9.9 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Biologicals (*Continued*)					
Tissue, plasma/ PL, PM, PN, PLP, PMP, PNP	Homogenize in water containing 4-deoxypyridoxine (IS), add 8% perchloric acid, centrifuge. Adjust to alkaline pH, stand and adjust pH to 5.2. Extract aliquot w/equal volume CH_2Cl_2, centrifuge. Add 2 mL 0.055 M HCl to 2 mL supernatant, autoclave, 5h, 121°C, cool, adjust pH to 5.7	μBondapak ODS, 10 μm, 30 cm × 3.9 mm Mobile phase—isocratic A—MeOH:water (85:15) B—1% HAC containing 0.005 M heptane sulfonic acid. Flow rate—1.5 mL min^{-1}	Fluorescence Ex λ = 300 Em λ = 375	%Recovery—82–96	*J. Chromatogr.*, 306, 377, 1984;[117] 374, 155, 1986[118]
Plasma, tissue, erythrocytes, lymphocytes/ PL, PLP	Add 50 μL MPCSM (IS) and 0.05 mL 10% TCA to 1 mL plasma, mix. Centrifuge. Add 50 μL 0.5 M semicarbazide to clear supernatant. Incubate, 4°C, 10 min, extract w/3 mL EtO_2	Partisil-10 ODS-3, 25 cm × 4.6 mm Partisil-5 ODS-3, 10 cm × 4.6 mm Mobile phase—isocratic 0.05 M KH_2PO_4, pH 2.9, containing 7% MeCN Flow rate—1.1 mL min^{-1}	Postcolumn alkalinization Fluorescence Ex λ = 367 Em λ = 478	QL—0.25 ng mL^{-1} $\%CV_{intra}$—5.9–8.1 %Recovery—92.3	*J. Chromatogr.*, 342, 277, 1985;[119] 375, 399, 1986;[120] Am. *J. Clin. Nutr.*, 46, 78, 1987;[121] 57, 47, 1993;[122] *J. Lab. Clin. Med.*, 113, 15, 1989[123]
Plasma, tissue, urine/PL, PM, PN, PLP, PMP, PNP, 4-PA	Add 5% MPA and 4-deoxypyridoxine (IS). Homogenize, centrifuge. Extract w/CH_2Cl_2, filter	Ultranex C_{18}, 3 μm, 15 cm × 4.6 mm Mobile phase—gradient A—0.033 M 1-octane sulfonic acid, pH 2.2 B—0.033 M H_3PO_4:IPA (IS), pH 2.2	Fluorescence Ex λ = 328 Em λ = 393	DL (on column)—0.1–0.4 ng QL—1.7–6.8 pmol %Recovery—86.3–111	*Nutr. Res.*, 9, 259, 1989[124]
Plasma, erythrocytes/ PL, PM, PN, PMP, PLP as 6-PA phosphate	Add 1 mL 3 M perchloric acid to 2 mL sample, vortex, and centrifuge. Adjust pH to 3.5, dilute to 5 mL w/water. Centrifuge, divide into two parts, inject one part directly into HPLC, the other part	TSK gel ODS-120A, 25 cm × 4.6 mm Mobile phase—isocratic 1% MeCN, 01 M sodium perchlorate—0.1 M KH_2PO_4, pH 3.5, 30°C Flow rate—0.5 mL min^{-1}	Fluorescence Ex λ = 305 Em λ = 390 4-PA-P Ex λ = 320 Em λ = 420	—	*Agric. Biol. Chem.*, 52, 1083, 1988;[125] *J. Nutr. Viaminol.*, 36, 521, 1990;[126] 40, 239, 1994;[127] *Meth. Enzymol.*, 230, 3, 1997[128]

	go through adjust pH, heat, readjust pH, stand, 24 h to convert PLP to PIC-P and inject				
Plasma/PLP, PA	Add potassium phosphate buffer (100 µL, 10 mM, pH 7.4), 200 µL (10%, w/v) to 100 µL sample. Heat, 50°C, 15 min, cool. Add dipotassium phosphate (140 µL, 3.3 M, pH 10.2), potassium cyanide (40 µL, 8 mM), mix. Heat, 50°C, 25 min, cool. Add H_3PO_4 (50 µL, 28%), centrifuge, inject supernatant	Apex™ 3 µm ODS, 25 cm × 4 mm Mobile phase—isocratic 1 mM heptane sulfonic acid—2 M potassium acetate (pH 3.75) Flow rate—1.0 mL min^{-1}	Fluorescence Ex λ = 318 Em λ = 418	%Recovery—92.8 $\%CV_{Intra}$—2.8 $\%CV_{Inter}$—5.2	*Eur. J. Clin. Nutr.*, 53, 448, 1999[129]
Plasma/PLP, 4-PA	Add 150 µL water to 50 µL plasma, mix. Add 200 µL TCA (100g L^{-1}), incubate, 50°C, 5 min. Add 70 µL K_2HPO_4 (3.3 M) and 20 µL sodium cyanide (0.04 M) to 200 µL resulting supernate, keep at 50°C, 25 min. Add 25 µL ortho-phosphoric acid (2.86 M), mix	Waters Symmetry Shield RP8, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic 75 mM Semicarbazide—50 mM KH_2PO_4, pH 2.85 Flow rate—1.5 mL min^{-1}	Fluorescence Ex λ = 325 Em λ = 418	%Recovery PLP—97.9–102.8 4-PA—97.6–101.9	*Clin. Chim. Acta*, 280, 101, 1999[130]
Plasma/PLP, PL, PMP, PN, PM, 4-PA	Add 10% ice cold metaphosphoric acid (v/v, 1:1), mix and centrifuge. Centrifuge and filter supernatant through 0.22 µm cellulose acetate filter	ODS, 3 µm, 15 cm × 4.6 mm Mobile phase—gradient 1.2 mM 1-octane sulfonic acid—0.033 M TEA (pH 2.16):MeCN Flow rate—1.2 mL min^{-1}	Postcolumn derivatization Fluorescence Ex λ = 328 Em λ = 393	%Recovery—70–110	*Anal. Biochem.*, 305, 82, 2002[131]
Plasma, red cells/PL, PLP, 4-PA	Add 40 µL semicarbazide and glycine (250 mg mL^{-1}) to 500 µL plasma or diluted red cell, vortex, incubate, room temperature, 30 min. Add 40 µL 70% perchloric acid, mix, centrifuge. Add 30 µL 25% NaOH to 300 µL supernatant, final pH 3–5	Luna C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic 60 mM Na_2HPO_4:MeOH (90.5:9.5) containing 400 mg L^{-1} EDTA disodium salt. Flow rate—1.5 mL min^{-1}	Fluorescence Ex λ = 380 Em λ = 450 4-PA-P Ex λ = 320 Em λ = 420	DL—1–2.8 nM QL—2.5–6.5 nM %Recovery—89–98 $\%CV_{Intra}$—3.0–12 $\%CV_{Inter}$—4.3–14	*J. Chromatogr. B*, 792, 333, 2003[132]

Continued

Table 9.9 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Biologicals (*Continued*)					
Cinchona bark/ PN-HCl	Dilute 300 mg sample to 50 mL of MeOH:mobile phase (70:30), ultrasonicate, 3 min. Dilute aliquot 1.5 to 5 mL w/mobile phase	Phenomenex Prodigy ODS, 5 µm, 25 cm × 3.2 mm Mobile phase—isocratic 0.05 M TEA phosphate buffer (pH 3.0):MeCN (88:12) Flow rate—0.4 mL min^{-1}	Fluorescence Ex λ = 330 Em λ = 420	DL—460 fM	*Anal. Chim. Acta*, 512, 85, 2004[133]
Serum/PLP, 4-PA	Add 150 µL cold metaphosphoric acid (5%) to 150 µL sample, mix and centrifuge. Add 150 µL CH_2Cl_2 to supernatant, vortex and centrifuge. Filter the top layer	Hypersil BDS C_{18}, 5 µm, 130-Á, 15 cm × 3 mm Mobile phase—gradient A—50 mM sodium phosphate (pH 3.1) containing 0.2% MeCN B—MeOH Flow rate—0.7 mL min^{-1}	Fluorescence Ex λ = 325 Em λ = 425	DL—0.3 nM QL—0.9 nM $\%CV_{Intra}$—0.6–1.8 $\%CV_{Inter}$—3.6–6.7 %Recovery—97–102	*Anal. Biochem.*, 333, 336, 2004[134]
Serum/PLP, 4-PA	Add 150 µL MPA (5%) to 150 µL sample, mix and centrifuge. Add 150 µL CH_2Cl_2 to supernatant, vortex and centrifuge. Filter the aqueous (top)	Hypersil BDS C_{18}, 5 µm, 15 cm × 3 mm Mobile phase—gradient A—50mM sodium phosphate (pH 3.1) containing 0.2% ACN B—MeOH Flow rate—0.7 mL min^{-1}	Postcolumn derivatization Fluorescence Ex λ = 325 Em λ = 425	DL—0.3 nM L^{-1} QL—0.9 nM L^{-1} %CV—0.9–6.7 %Recovery—97–101	*Anal. Biochem.*, 333, 336, 2004[134]
Serum, standard/ PLP, PL, 4-PA	—	Phenomenex silica-based SCX, 10 µm, 25 cm × 4.6 mm	Postcolumn derivatization	DL—1.6 nM for PLP %Recovery—95–105	*J. Chromatogr. B*, 823, 218, 2005[135]
Plasma/PMP, PLP, PNP, 4-PA, PL, PM, PN	Add 60 µL TCA (50 g L^{-1}) to 60 µL sample, mix, stand in ice, 60 min. Centrifuge, inject supernatant	Agilent stable-bond C_8 5 µm, 12.5 cm × 4.6 mm Mobile phase—gradient A—0.65 M HAC B—0.1 M heptafluorobutyric acid in 0.65 M HAC C—90% ACN in water Flow rate—1 mL min^{-1}	Mass spectrometer	DL—0.1-4.0 nM %CV—3–22 %Recovery—78–163	*Clin. Chem.*, 51, 1206, 2005[136]

procedure appears to be an excellent extraction for the three vitamins and can save considerable time, compared to single vitamin extractions.

Various extractions for food and serum are summarized in Table 9.9.

9.3.2.2.2 Chromatography parameters

Supports and Mobile Phases

Liquid chromatography methods developed in the early 1970s largely relied upon ion-exchange resins while more recent methods have used C_{18} reversed-phase systems with ion-pairing agents. Vanderslice et al.[96] successfully resolved the six vitamers and 4-PA on Aminex A-25 anion-exchange resin. The procedure was based upon a complicated instrumentation setup and laboratory packed columns and was not extensively used by other researchers. However, the Vanderslice procedure and a series of subsequent papers adapting the procedure to food and serum analysis provided great insight into analysis of biological matrices by LC.[97–101,112] Two important developments resulted from the Vanderslice group's in-depth look at vitamin B_6 analysis. Vanderslice et al.[99] introduced and proved the worth of sulfosalicylic acid (SSA) for food extraction. Sulfosalicylic acid was subsequently used in many other studies as the extractant. Vanderslice and Maire[97] added 0.005 M semicarbazide into the anion-exchange elution buffer to increase the fluorescence response of PL and PLP by conversion of the compounds to the oxime. This technique increased the fluorescence of the PL and PLP to levels comparable to the other vitamers. Application of the chromatography system developed by Vanderslice and colleagues was hampered by manufacturing changes in the Aminex A-25 resin that altered resolution of the vitamers.

Methods following Vanderslice's developmental work are simpler, less time-consuming, and largely based on reversed-phase systems. However, Coburn and Mahuren[115] used cation-exchange chromatography on Vydac 401TP-B that resolved the vitamin B_6 vitamers and 4-PA. Important to the development of later procedures, the method introduced the postcolumn use of a potassium phosphate buffered (pH 7.5) bisulfite solution to enhance the fluorescence of PLP. The buffered solution allowed the detection of PNP, which was not fluorescent in the acid mobile phase at the fluorescence wavelengths (Ex $\lambda = 330$, Em $\lambda = 400$) required for the other analytes. The postcolumn treatment permitted the detection of the six vitamers, 4-PA, and the internal standard (2-amino-5-chlorobenzoic acid) at the same wavelengths. After the methodology was introduced, chromatography parameters were slightly modified for quantitation of other vitamin B_6 metabolites. These include 5-pyridoxic acid, 5-pyridoxic acid lactone, pyridoxo-4:5-lactone, and pyridoxo-5:4-lactone.[116] The authors state that cation-exchange LC is better suited to resolution of the urinary metabolites than ion-pair, reversed-phase chromatography. The Vydac 401TP-B support is no longer available and was replaced with Nucleosil 5 SA.[116]

Reversed-phase chromatography on C_{18} has become the most common approach to vitamin B_6 analysis. As shown in Table 9.9, compositions of the mobile phases vary considerably, but most incorporate ion-pairing reagents to optimize resolution. Both gradient and isocratic systems successfully resolve the six vitamers and 4-PA as well as different internal standards used by the various investigators. One of the earliest applications of reversed-phase chromatography for vitamin B_6 was published by Gregory and Kirk.[139] Further method development by Gregory and colleagues was instrumental to development of current approaches for vitamin B_6 analysis. Important contributions included conversion of PLP to the semicarbazone in tissue and plasma,[111] conversion of PMP and PM to PLP and PL by deamination with glyoxylate before derivatization with semicarbazide,[140] and development of procedures to assay PN-glucoside.[35,141] The Gregory and Ink[35] procedure for identification and quantitation of PN-glucoside is summarized in Section 9.4. This method is highly significant to the accurate measurement of biologically active vitamin B_6 in plant foods.

Ubbink et al.[119,120] adapted Gregory's semicarbazide enhancement of the fluorescent response of PLP to plasma and tissue analysis. Their studies showed that the semicarbazones of PL and PLP were stable under conditions that rapidly decomposed free PL and PLP. The semicarbazide derivatization agent was directly added to the extraction solution, simplifying previous precolumn and postcolumn derivatization procedures. Later studies proved the utility of TCA as a deproteinizing agent for plasma and tissue analysis. Because of the significance to plasma analysis of the Ubbink procedure, it is provided in Section 9.4.

Reitzer-Bergaentzle et al.[52] reported an improved procedure for the analysis of total vitamin B_6 in foods using ion-pair chromatography. The method incorporates a precolumn transformation of phosphorylated and free vitamin B_6 forms into pyridoxol. Acid phosphatase hydrolysis is used for dephosphorylation followed by desamination with glyoxylic acid in the presence of Fe^{2+} to convert pyridoxamine into pyridoxal. Pyridoxal is then reduced by sodium borohydride to pyridoxol. The method provided recoveries between 90% and 95%, and a detection limit of 0.02 μg g^{-1}. Following initial reporting of the procedure, a collaborative study was completed that included 12 European laboratories.[53] Analysis of eight different samples gave RSD_R values of 12%–13% for samples with labeled or reference vitamin B_6 contents and 30%–35% for foods with low vitamin levels. Values from four laboratories that used microbiological analysis agreed with the LC results. Mann et al.[50] evaluated the Reitzer-Bergaentzle method for the analysis of total vitamin B_6 in soy-based infant formula. Success was excellent and the procedure was collaborated by AOAC International for assay of soy- and milk-based infant formulas in 2004.[51] Procedural aspects of AOAC Method 50.1.26 (2004.07) "Vitamin B_6 in Reconstituted Infant Formula"[45] are provided in Section 9.4.

9.3.2.2.2 Detection and enhancement of PLP fluorescence. UV detection is not usable for analysis of biological samples. $E^{1\%}_{1\text{cm}}$ values (Table 9.7) range from less than 10 to slightly above 400 depending upon the vitamer and solvent environment, indicating a relatively low UV detection sensitivity. For this reason, fluorescence is the universal detection mode unless highly fortified foods, medical foods, or pharmaceutical products are under study. Methods used to enhance the fluorescence of PL and PLP developed by the leading research groups on vitamin B_6 analysis include enhancement by formation of a bisulfite adduct,[97,115,116,131,142,143] formation of semicarbazones by reaction with semicarbazide,[111,113,119–123,132] and oxidation by cyanide to carboxylic acid.[125–128,130,144–147] Bisp et al.[131] used the bisulfite derivatization for the analysis of vitamin B_6 and pyridoxic acid in plasma (Figure 9.5). A more recent application of precolumn semicarbazide derivatization was by Talwar et al.[132]

In recent publications,[134,135] use of cyanide has been eliminated by postcolumn conversion of PLP to 4-pyridoxic acid 5′-phosphate by chlorite. The method is based on original work by Lindgren and Nilsson.[148] Rybak and Pfeiffer[134] and Ericson et al.[135] increased sensitivity 4× using the chlorite derivatization compared to bisulfite adduct formation.

Rybak et al.[149] compared LC methods using PLP enhancement techniques to nonradiometric enzymatic assays commonly used in clinical laboratories. The study included ten clinical laboratories—all experienced in serum vitamin B_6 analysis. The general conclusion reached by the authors stated, "Agreement among vitamin B_6 methods is good, but large differences in laboratory proficiency exist, pointing to the need for vitamin B_6 reference materials and external quality assurance programs." Mean laboratory within-day % coefficient of variation (% CV) ranged from 0.6% to 37%. Between-day % CV values ranged from 1.4% to 26%. Five of the laboratories using LC methods and one using an enzymatic assay reported results that satisfied the 99% probability inclusion criteria for the consensus mean

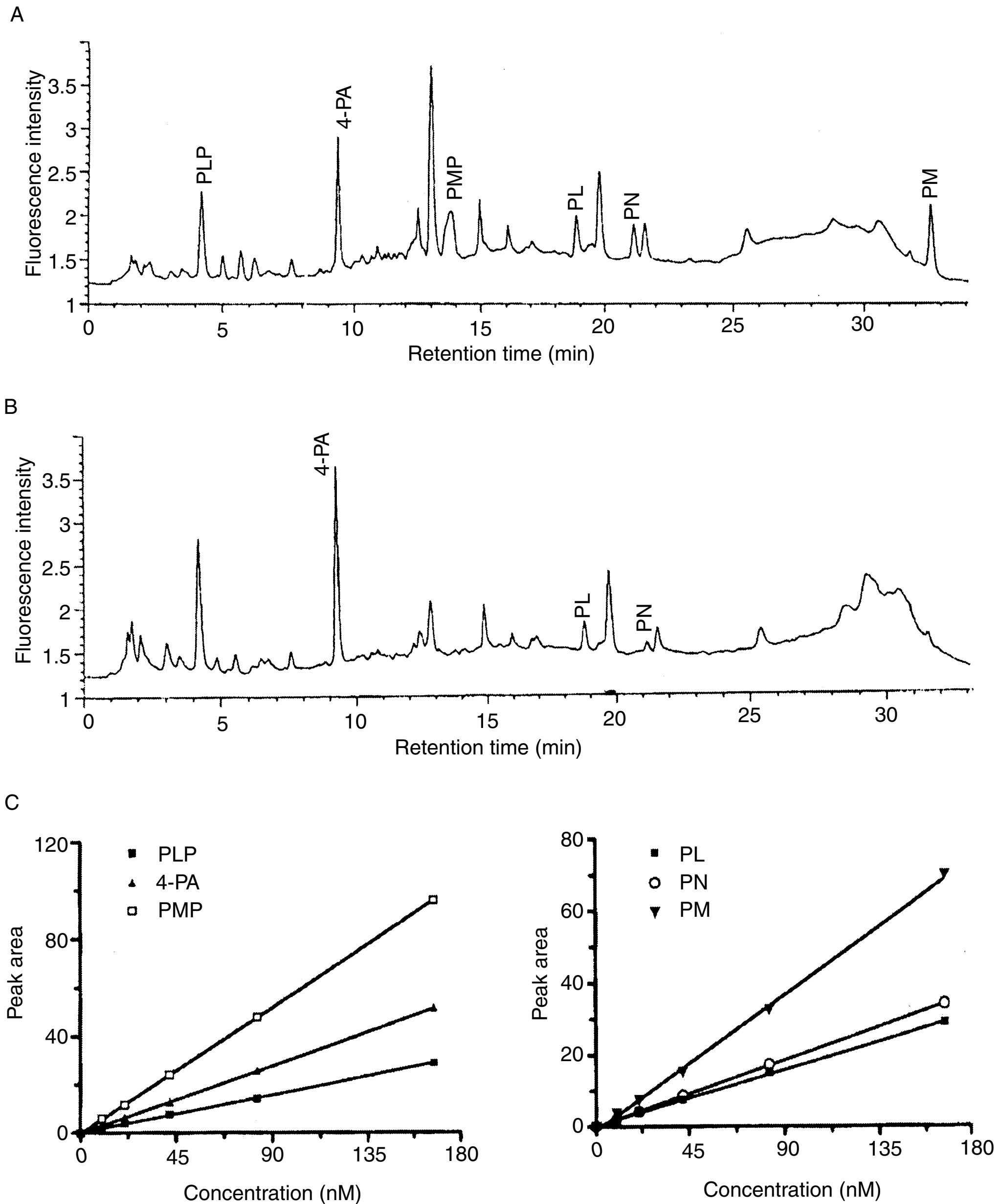

Figure 9.5 HPLC chromatograms for spiked (24.7 mM of each vitamin B_6 vitamer and 4-PA) (A) and unspiked (B) plasma (C) calibration curves (Reproduced from Bisp, M. R., Bor, M. V., Heinsvig, E., Kall, M. A., and Nexø, E., *Anal. Biochem.*, 305, 82, 2002. With permission.)

calculations of all serum pools. The authors suggested that quality control and calibration accuracy were the two largest problems. Further, they suggested that development and validation of reference methods such as isotope-dilution LC-MS/MS capable analyte confirmation were needed.

At the time of preparation of the current edition, few methods exist that use the resolution power of liquid chromatography matched with the selectivity and sensitivity of

mass spectrometry for vitamin B_6 analysis. As with all of the vitamins, LC-MS or LC-MS/MS (liquid chromatography-tandem mass spectrometry) has the potential to improve accuracy. Midttun et al.[136] introduced a LC-MS/MS method for the quantification of all known forms vitamin B_6 and riboflavin in human plasma at the nanomole level. This research is discussed more in-depth in Chapter 14. Gas chromatography (GC) is used only infrequently for vitamin B_6 analysis. However, Nùñez-Vergara et al.[150] presented a gas chromatography-mass spectrometry (GC-MS)-based method for the simultaneous assay of melatonin and PN after derivatization with *N*-methyl-*N*-*N*-trimethylsilytrifluoroacetamide.

Internal Standards

Owing to the complexity of the chromatography, vitamin B_6 analysis of nonfortified foods and other biological samples should not be attempted without the use of a suitable internal standard. Gregory[41] reviewed the use of several compounds that have similar properties to the vitamin B_6 forms commonly found in biological samples. These include 4-deoxypyridine, 3-hydroxypyridine, 6-methyl-2-pyridine carboxaldehyde, and 2-amino-5-chlorobenzoic acid. Of these, 4-deoxypyridine has been used most frequently. Addition of 4-deoxypyridine at levels required to provide sufficient fluorescence response can interfere with elution of low level metabolites in some systems.[128] Deuterated standards (d_2-PLP, d_3-PL) were used in the LC-MS/MS method of Midttun et al.[136]

9.4 Method protocols

Stability of Pyridoxal-5-Phosphate Semicarbazone: Applications in Plasma Vitamin B_6 Analysis and Population Surveys of Vitamin B_6 Nutritional Status

J. Chromatogr., 342, 277, 1985[119]

Principle

Pyridoxal (PL) and PLP are converted precolumn to their respective semicarbazones. Vitamers are resolved isocratically on Partisil 10 ODS-3 and detected by fluorescence. Internal standard is 6-methyl-2-pyridine carboxaldehyde semicarbazone.

Chemicals

- PL, PLP
- 6-Methyl-2-pyridine carboxaldehyde (IS)
- CH_2Cl_2
- Acetonitrile
- Methanol
- Glacial acetic acid
- Semicarbazide

Apparatus

- Liquid chromatograph
- Fluorescence detector

Procedure

Extraction

- Add 50 mL reconstituted 6-methyl-2-pyridine carboxaldehyde semicarbazone (IS) to 1 mL plasma.
- Add 0.5 mL 10% TCA.

- Mix, centrifuge.
- Add 50 mL of 0.5 M semicarbazide to clear supernatant.
- Incubate, 40°C, 10 min.
- Extract, 2 3 with 3 mL ethyl ether.
- Aspirate ethyl ether, extract water phase with 3 mL CH_2Cl_2.

Chromatography

See Figure 9.6.

Column	25 cm × 4.6 mm
Stationary phase	Patisil 10 ODS-3
Column temperature	Ambient
Mobile phase	0.05 KH_2PO_4 with 7% acetonitrile, pH 2.9
Flow rate	1.1 mL min^{-1}
Postcolumn reagent	4% NaOH, 0.1 mL min^{-1}
Detection	Fluorescence Ex λ = 367, Em λ = 478
Calculation	Internal standard 6-methyl-2-pyridine carboxaldehyde semicarbazone

Identification and Quantification of Pyridoxine-β-Glucoside as a Major Form of Vitamin B_6 in Plant-Derived Foods

J. Agric. Food Chem., 35, 76, 1987[35]

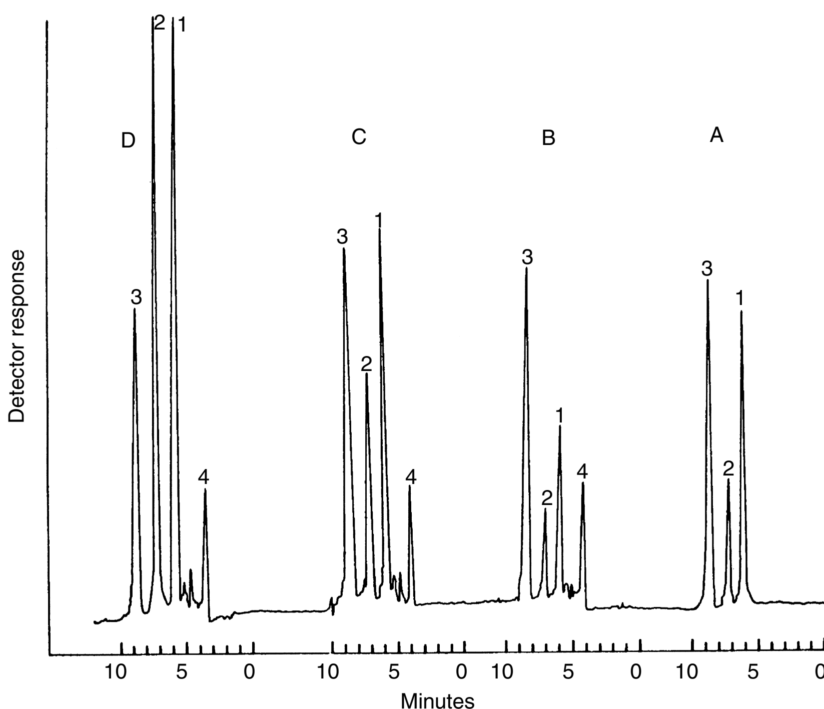

Figure 9.6 PL and PLP in plasma: (A) standard, (B) normal plasma, (C) plasma spiked with PL and PLP, and (D) plasma, subject on oral supplements. Peak 1—PLPSC, Peak 2—PLSC, Peak 3—MPCSC (IS), Peak 4, unknown. (Reproduced from Ubbink, J. B., Serfontein, W. J., and DeVilliers, L. S., *J. Chromatogr.*, 342, 277, 1985. With permission.)

Principle

Extract product with sulfosalicylic acid (SSA). Sample extracts are treated by anion-exchange chromatography to remove SSA. Vitamin B_6 vitamers and pyridoxine-β-glucoside are quantitated by reversed-phase chromatography and fluorescence detection following postcolumn enhancement of PL and PLP by buffered bisulfite.

Chemicals

- Hydrochloride salts of PN, PL, PM, and 4-deoxypyridoxine
- Pyridoxal-5′-phosphate
- 4-pyridoxic acid
- Pyridoxamine-5′-phosphate
- Isopropanol
- Sulfosalicylic acid
- l-octane sulfonic acid (sodium)

Apparatus

- Liquid chromatograph
- Fluorescence detector
- Gradient controller
- Anion-exchange column, AG2-×8 resin

Procedure

Extraction

- Mix 2 g sample with 9 mL 5% SSA.
- Homogenize with Polytron, 45 s, setting 7.
- Add 10 mL CH_2Cl_2.
- Blend.
- Centrifuge.
- Remove aliquot of aqueous layer.

Preparative Anion-Exchange Clean Up

- Inject 0.3–0.4 mL extract onto preparative LC column of AG2-×8 resin by injecting into flowing stream of 0.1M HCl.
- SSA is bound to resin.
- Monitor vitamin B_6 elution by fluorescence.
- Collect effluent (6–10 mL).
- Neutralize with 1 M NaOH.

β-Glucoside Treatment

- Dilute 0.9 mL of eluate with 0.1 mL 1 M sodium phosphate, pH 5.
- Add 0.05 mL of β-glucoside (2 mg in water).
- Incubate, 37°C, 5 h.
- Deproteinate with 0.07 mL 100% TCA.

Chromatography

See Figure 9.7.

Stationary phase	Perkin-Elmer ODS
Column temperature	Ambient

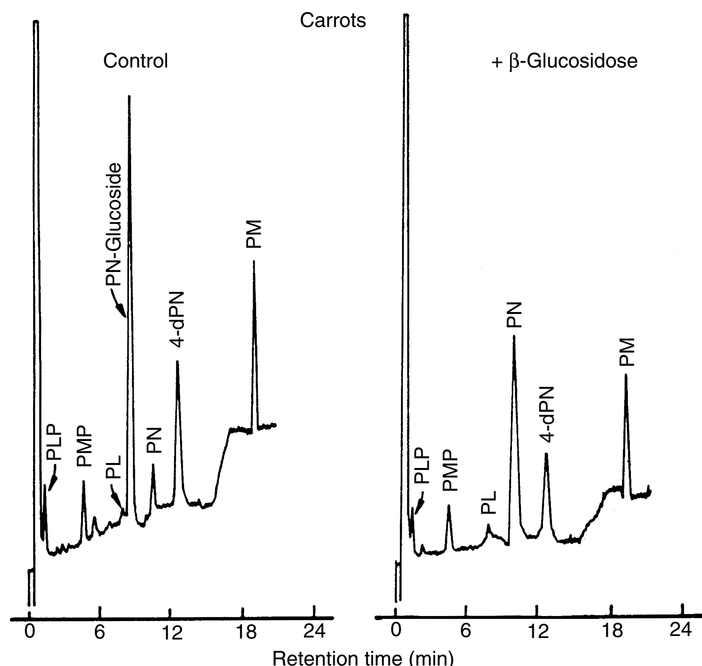

Figure 9.7 PN-glucoside chromatography before and after β-glucosidase treatment. (Reproduced from Gregory, J. F., III, and Ink, S. L., *J. Agric. Food Chem.*, 35, 76, 1987. With permission.)

Mobile phase	A1—0.033 M KH_2PO_4 and 8 mM octanesulfonic acid, pH 2.2
	A2—0.033 M KH_2PO_4, 8 mM octanesulfonic acid, and 2.5% isopropanol, pH 2.2
	B—0.033 M KH_2PO_4, 6.5% isopropanol, pH 2.2
Gradient	100% A1 to 100% A2 in 12 min
	To 100% B in 15 min after injection
	Equilibration requires 10 mm after elution of PM
Flow rate	1.8 mL min^{-1}
Detection	Fluorescence
	Ex λ = 330, Em λ = 400
Calculation	Internal standard, 4 deoxypyridoxine

Liquid Chromatographic Analysis of Vitamin B_6 in Reconstituted Infant Formula: Collaborative Study

J. AOAC Int., 84, 1593, 2001;[50] *J. AOAC Int.*, 88, 30, 2005[51]

Principle

Vitamin B_6 is dephosphorylated by enzymatic hydrolysis. Simultaneously, reaction with glyoxylic acid in presence of Fe^{+2} catalyst, pyridoxamine is transformed into pyridoxal, which is subsequently reduced to pyridoxine by the action of sodium borohydride in the alkaline medium. The total vitamin B_6 is quantified by ion-pair liquid LC.

Apparatus

- Liquid chromatograph
- Fluorescence detector
- Constant temperature incubator shaker

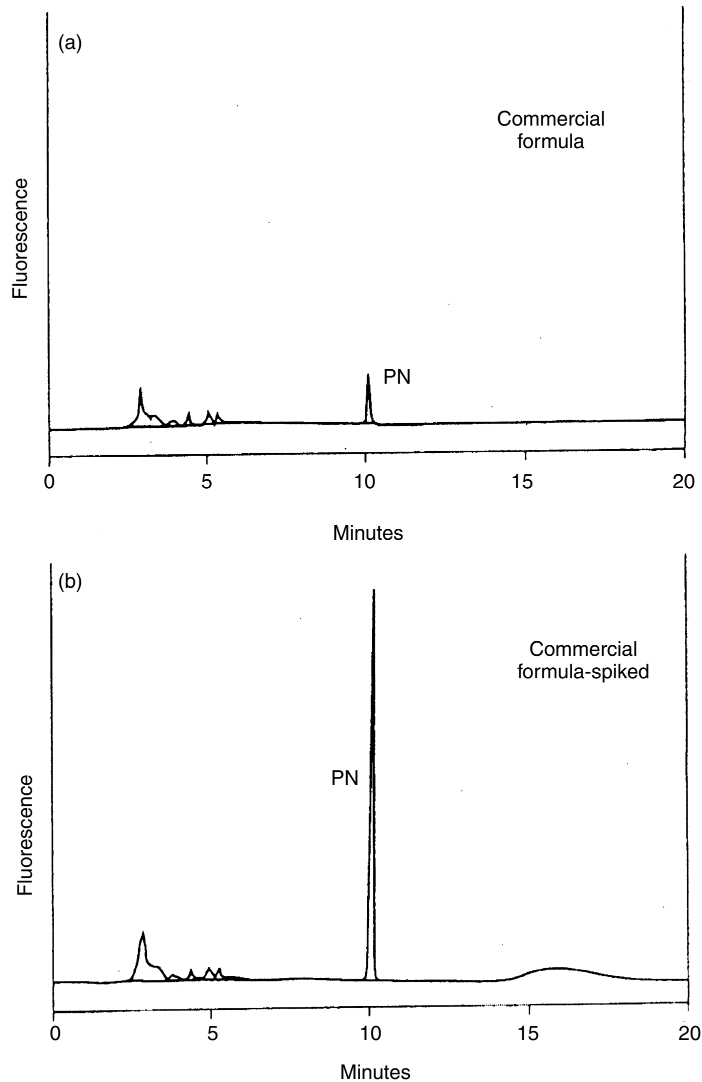

Figure 9.8 Chromatograms of commercial infant formula (a) before spiking and (b) after spiking with pyridoxamine and with conversion to pyridoxine (PN). (Reproduced from Mann, D. L., Chase, G. W., Jr., and Eitenmiller, R. R., *J. AOAC Int.*, 84, 1593, 2001. With permission.)

Chemicals

- Pyridoxamine—2HCl standard
- Sodium hydroxide
- Sodium borohydride
- 1-Heptane sulfonic acid sodium salt
- Potassium dihydrogen phosphate
- Orthophosphoric acid
- Pyridoxal—HCl standard
- Pyridoxol—HCl standard
- Acid phosphatase

- Sodium acetate
- Glacial acetic acid
- Glyoxylic acid
- Ferrous sulfate

Extraction and Derivatization

Preparation of test sample" Weigh 25 g well-mixed, ready-to-feed infant formula into 50 mL Erlenmeyer flask. For powdered infant formulas, dilute accordingly to manufacturer's instruction (12–15 g 100 mL^{-1}).

Derivatization: Add the following reagents to the test solutions and working standards in the following order: 2.0 mL 0.625M sodium acetate solution; 2.5 mL 1M glyoxylic acid reagent; 0.8 mL 10 g L^{-1} ferrous sulfate solution; 1.0 mL 20 mg mL^{-1} acid phosphatase solution.

- Add four 6 mm glass beads to all test solutions to ensure mixing during incubation.
- Place solutions in a shaker bath at 37°C overnight (or at least 12 h) to ensure complete dephosphorylation of the analytes. Remove the incubated extracts from the shaker. Let cool and quantitatively transfer to 50 mL volumetric flasks.
- Dilute to volume with deionized water and mix. Filter extract through Whatman No. 40 filter paper, or equivalent.
- Discard first 10 mL of extract. Transfer 5.0 mL clear filtrate to a small flask or beaker; add 4.5 mL 0.1M sodium borohydride solution. Mix by gently swirling for ca. 20 s. Add 0.5 mL glacial acetic acid. Gently swirl for 30 s. After effervescence has subsided, filter ca. 2 mL final extract through a 0.45 μm syringe filter.

Chromatography

See Figure 9.8 (Reference 50)

Column	25 cm × 4.6 mm
Support	Luna phenyl-hexyl, 5 μm
Mobile phase	Methanol–0.01 M (26 + 74) with 1 g 1-heptane sulfonic acid per 2 L, pH 2.5–2.6
Flow rate	1.0 mL min^{-1}
Injection volume	20 μL
Detection	Fluorescence Ex λ = 290, Em λ = 395
Calculation	External standard, linear regression

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chapter ten

Folate and folic acid

10.1 Review

Folate deficiency was recognized by Wills in 1931 through identification of a pernicious anemia prevalent in Indian women. The deficiency was inducible in monkeys that were fed the same diet and could be alleviated by feeding yeast or liver extracts. The antianemia factor was designated "vitamin M" in 1938.[1] In the following year, a similar anemia, curable by liver extract, was reported in chicks.[2] The name "folic acid" was first used by Snell's group in studies on the growth factors for *Lactobacillus casei* and *Streptococcus lactis*. Folic acid was isolated in quantity from spinach, and the name was derived from the Latin word "folium" for leaf.[3] The *L. casei* growth factor was purified and synthesized through the efforts of several biochemists (1941–1948). An excellent review of the history of folic acid research is provided by Friedrich.[4]

Identification of folic acid as a cure for megaloblastic anemia occurred in 1945.[5] Since then, folate deficiency is recognized as one of the most prevalent vitamin deficiencies common in all areas of the world. Deficiency results from inadequate intake, defective absorption, abnormal metabolism, or conditions such as drug therapy, leading to increased requirements.[5] Marginal deficiency produces general symptoms including tiredness, irritability, and decreased appetite. Severe deficiency produces megaloblastic anemia or the production of large immature red blood cells. Other symptoms include abdominal pain, diarrhea, ulcers in the mouth and pharynx, skin changes, hair loss, and neurological disorders such as dementia and depression.[5,6]

Close relationships between folate and vitamin B_{12} deficiencies result from the involvement of the vitamins in DNA synthesis. Megaloblastic anemia produced by deficiencies of folate and vitamin B_{12} are characterized by abnormally large megaloblasts in the bone marrow and abnormally large red cells in the blood.[6] Impaired DNA synthesis arises from decreased production of 5,10-methylene tetrahydrofolate (5,10-CH_2H_4 folate), which is required for the synthesis of deoxythymidine monophosphate nucleotide.[7] Similarities in the megaloblastic anemias produced by deficiencies of vitamins require biochemical assessment for accurate diagnosis and differentiation of the causative vitamin. Plasma folate levels (5-CH_3-H_4 folate) rapidly fluctuate with recent intake; therefore, erythrocyte folate levels are considered a more reliable status index.[6] Serum folate levels stabilize after a few weeks at about 7 nmol L^{-1} when subjects are on a folate-deficient diet,[8] indicating acute status but not the level of tissue stores.[6] Erythrocyte folate levels follow liver levels and reflect tissue stores. The Institute of Medicine, in consideration of Dietary Reference Intake (DRI) values for folate, used an erythrocyte level of <305 nmol L^{-1} as the indicator of inadequate status.[9] An indirect clinical measure is the urinary excretion of formiminoglutamic acid (FIGLU).

FIGLU is an intermediate in the conversion of histidine to glutamic acid by a folate-requiring enzyme—formiminotransferase. Folate deficiency results in lowered enzyme activity and increased FIGLU excretion.[6] Gibson[6] indicates that normal urine FIGLU levels are <35 μmol 24 h^{-1} with an increase to <144 μmol 24 h^{-1} after a 15 g histidine load. In folate-deficient individuals, the 15 g histidine load increases FIGLU excretion to 200–11,500 nmol 24 h^{-1}. Unfortunately, increased excretion of FIGLU occurs under other metabolic disturbances and is not considered specific enough for completely accurate clinical diagnosis of folate deficiency. The test is, therefore, infrequently used.

With the recognition of the strong relationship between folate status and the occurrence of neural tube defects including spina bifida and the relationship of elevated serum homocysteine levels with increased statistical chance of development of coronary heart disease,[10,11] global interest in folate nutrition has dramatically increased. In order to assist women of childbearing age to increase folate intake and reduce the risk of a pregnancy affected by a neural tube birth defect, the U.S. Food and Drug Administration finalized regulations in 1993 that mandated fortification of enriched cereal grain products with folic acid.[12–16] Fortification levels (Table 10.1)[17] were set to help ensure that women of childbearing age meet the U.S. Public Health Service recommendation of an intake of 400 μg folate per day.[18,19] Since the fortification policy became mandatory on January 1, 1998, serum and erythrocyte folate concentrations in all sex and age groups has dramatically increased. Two recent publications by Pfeiffer et al.[20] and Dietrich et al.[21] provide comparisons between the National Health and Nutrition Examination Surveys (NHANES III, 1988–1994—before fortification) and the 1999–2000 NHANES survey (after fortification). These studies show that the prevalence of low serum folate levels (<6.8 nmol L^{-1}) decreased from 16% before initiation to 0.5% after initiation of folic acid fortification.[20] In the elderly, the prevalence of high serum folate levels (≥45.3 nmol L^{-1}) increased from 7% before fortification to 38% after fortification.[20] Overall mean serum concentrations increased from 11.4 to 26.9 nmol L^{-1} (2.35×) and mean erythrocyte levels increased from 375 to 590 nmol L^{-1} (1.57×).[21] However, less than 10% of women of childbearing age reached the recommended erythrocyte folate concentration of >906 nmol L^{-1} that leads to a significant reduction in neural tube defect risk.[21] Results show that the U.S. population has greatly improved food folate status due to folic acid fortification of cereal grain. At the same time, use of supplemental folic acid is encouraged for women of childbearing age to further decrease neural tube risk.

Since the U.S. fortification program was initiated, many other countries including Canada, Mexico, Chili, and Hungary have started fortification programs.[22] The significance of improved folate status has greatly increased with the recognition that folate may play a role in reducing

Table 10.1 Fortification Levels of Cereal-Grain Products with Folic Acid

Enriched cereal-grain products	Folic acid μg 100 g^{-1}	Products fortified
Part 136 bakery products		
Enriched bread, rolls, buns	95	All
Part 137 cereal flours and related products		
Enriched flour	154	All
Enriched corn meals	154–220	All
Enriched farina	154–220	All
Enriched rice	154–308	All
Part 139 macaroni and noodle products		
Enriched macaroni products	198–264	All
Enriched noodle products	198–264	All

Source: Rader, J. I., Weaver, C. M., and Angyl, G., Use of a microbiological assay with trienzyme extraction for measurement of prefortification levels of folates in enriched cereal-grain products, *Food Chem.*, 62, 451, 1998.

the risk of many age-related, chronic diseases including cardiovascular diseases, macular degeneration, various cancers, and neurodegenerative disorders including stroke, Alzheimer's disease, and Parkinson's disease.[23,24] Lucock[23] traced research publications from 1992 to 2002 and showed the extent of expanded research interest in folate that exceeded that of most other vitamins during that decade. Rapid increase was noted in publications dealing with relationships of folate to vascular disease, cancer, birth defects, and pregnancy.[23]

Understanding of the interrelationships in metabolism of folate, methionine, and homocysteine and the recognition that an elevated serum homocysteine level is an independent risk factor for cardiovascular disease has been a highly significant area of recent research. Folate is required for the remethylation of homocysteine to methionine, which is dependent upon sufficient levels of 5-methyltetrahydrofolate (5-CH_3-H_4 folate) as the one-carbon donor (Figure 10.1). While high circulating levels of homocysteine is an independent risk factor for cardiovascular disease, other consequences of the disruption in methionine metabolism caused by insufficient 5-CH_3-H_4 folate include depletion of S-adenosylmethionine, which is the methyl donor for methyltransferase reactions.[23–33] Depletion of S-adenosylmethionine results in decreased methylation of cytosine in DNA, which can cause increased gene transcription, DNA strand breakage, and impaired DNA repair as well as decreased methylation of proteins, phospholipids, and neurotransmitters.[25] Lucock's[23] summation of current research on folate and health relationships concluded that recent emphasis on folate centers on the discovery of several single nucleotide polymorphisms that modulate risk for diseases associated with morbidity and mortality. Folate can interact with the proteins encoded by the variant genes and ameliorate risk.[23] Single nucleotide polymorphisms of genes associated with folate metabolism and with various clinical states are given in Table 10.2.

Folates are present in most foods with legumes (peanuts, cowpeas, peas, etc.), leafy greens, citrus (orange juice), some fruits, vegetables (broccoli, cauliflower), and liver considered to be good sources. Enriched cereal products are fortified at 140 μg 100 g^{-1}. The addition level is estimated to increase the intake of 19- to 50-year-old women to levels slightly below the Reference Daily Intake (RDI) of 400 μg.[34] Daly et al.[35] calculated the statistically expected reduction in risk of neural tube defects if women of childbearing age with low red cell folate levels consumed the RDI. At the RDI, an approximate 50% reduction in neural tube defect was expected. For this reason, the current addition level required by Food and Drug Administration (FDA) regulations is controversial. Higher levels of addition would further decrease the incidence of neural tube defect. However, the conservative FDA approach was dictated by the possibility that higher folate intake by the general population would mask vitamin B_{12} deficiency.[36] Table 10.3 provides folate content of foods in the U. S. diet compiled from the USDA National Nutrient Database for Standard Reference, Release 19.[37] As with most water-soluble vitamins that are added to breakfast cereals, folate concentrations are highest in these products. Products containing enriched cereal grains and the enriched flours are also high in the ranking. Unfortified foods including liver, legumes, okra, spinach, asparagus, and leafy greens are excellent food sources.

Dietary Reference Intake (DRI) values for folate include Recommended Dietary Allowance (RDA) values for children and adults. RDA's for adult men and women are 400 μg d^{-1} (Table 10.4).[9] Calculation of μg dietary folate equivalent (DFE) is discussed in Section 10.4.

Various attempts worldwide are underway to increase folate content of foods through bioprocessing and bioengineering studies. Jägerstad et al.[38] reviewed bioprocessing studies aimed at increasing folate content of bread, vegetables, dairy products, and beer through microorganism selection for fermented products, modification of malting processes, and process modifications for vegetables. Folate contents were increased in such products by several times. Fermentation changes resulted in up to a sevenfold increase in beer. Changes in milling procedures increased folate in flours up to 20-fold. Such refined

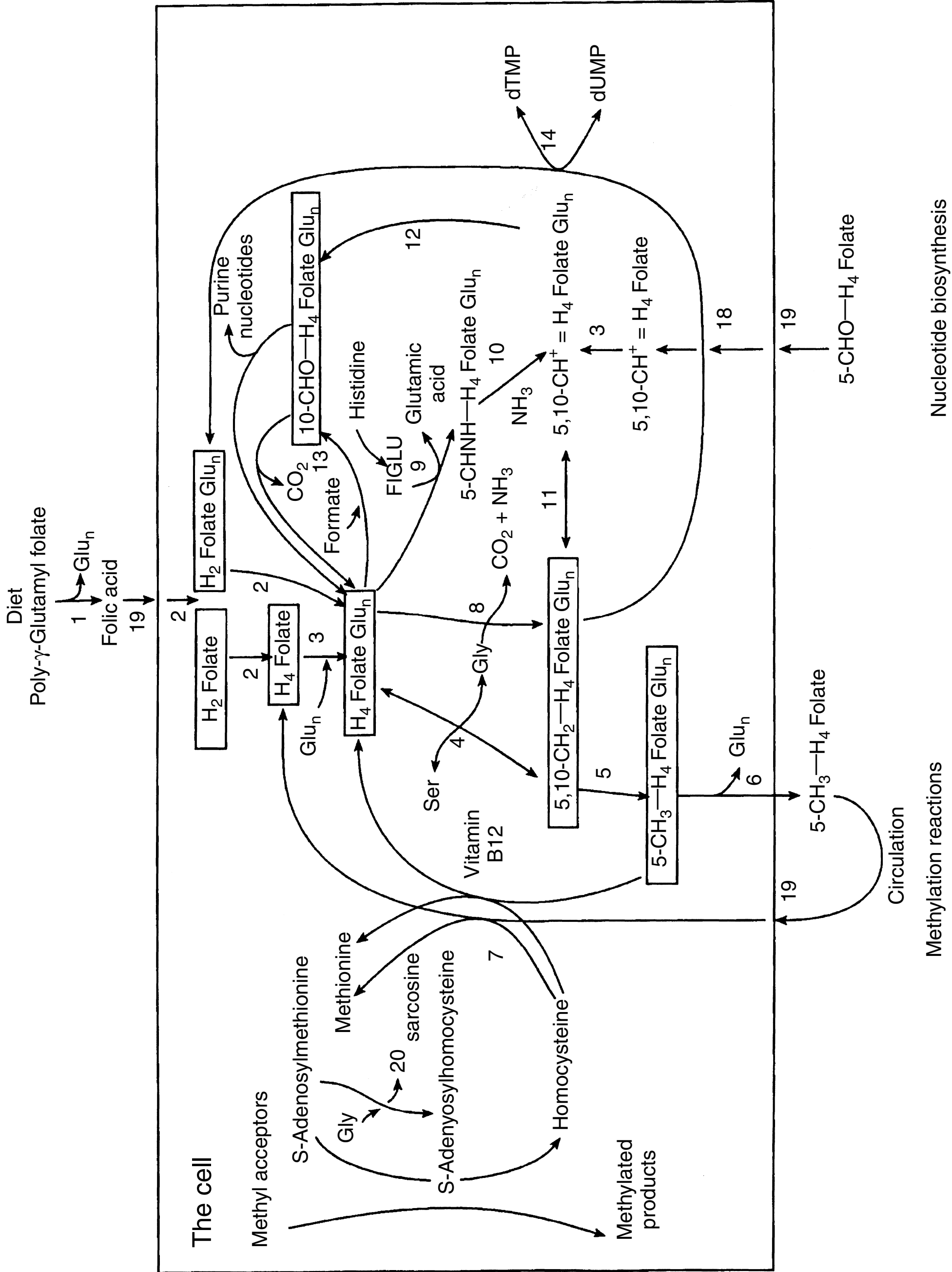

Figure 10.1 Metabolic interrelationships of the folates.

Table 10.2 Single Nucleotide Polymorphisms of Genes Associated with Clinical States

Variant	Gene	Association
C677T	5,10-CH_2H_4 folate reductase	Colon cancer
		Elevated homocysteine
		Spina bifida
		Down's syndrome
		Oral cleft
		Adult acute lymphocytic leukemia
		Pregnancy complications
A1298C	5,10-CH_2H_4 folate reductase	Spina bifida
		Adult acute lymphocytic leukemia
A2756G	Methionine synthetase	Thromboembolic vascular disease
		Homocysteine response to diet
A66G	Methionine synthetase reductase	Spina bifida
		Down's syndrome
C1561	γ-Glutamyl carboxypeptidase	Cardiovascular disease

Source: Lucock, M., Is folic acid the ultimate functional food component for disease prevention? *Br. Med. J.*, 328, 211, 2004.

procedures could greatly enhance folate levels in commonly consumed foods without use of enrichment.[39,40]

Enhanced folate levels in plant products through bioengineering is being actively pursued. Studies at the University of Florida[41] using a synthetic gene based on mammalian guanosine triphosphate (GTP) cyclohydrolase 1 increased pteridine (folate precursor) 140× in tomato fruit and the folate levels in the tomatoes by twofold. The authors attributed the folate increase to increased levels of 5-CH_3H_4 polyglutamates and 5,10-CH=H_4 polyglutamates. Readers interested in this approach to increased folate availability in the food supply are encouraged to read reviews by Basset et al.[42] and Storozhenko et al.[43]

Folate is the general term inclusive of folic acid (pteroylglutamate) and poly-γ-glutamyl conjugates that exhibit the biological activity of folic acid. Structural characteristics are discussed in Section 10.2. Folates function in single-C metabolism as acceptors or donors of single C-units (methyl, methylene, methenyl, formyl, and formimino groups). Metabolically significant forms include 7,8-dihydrofolate (H_2 folate), 5,6,7,8-tetrahydrofolate (H_4 folate), 5-methyltetrahydrofolate (5-CH_3–H_4 folate), 5-formyltetrahydrofolate (5-CHO–H_4 folate), 10-formyltetrahydrofolate (10-CHO–H_4 folate), 5,10-methenyltetrahydrofolate (5,10-CH^+ u H_4 folate), 5,10-methylene-tetrahydrofolate (5,10-CH_2–H_4 folate), 5-formiminotetrahydrofolate (5-CHNH–H_4 folate), and their respective γ-glutamyl conjugates. Metabolic interrelationships of the biologically significant forms are provided in Figure 10.1.[44,45] Enzymes indicated by numerals in Figure 10.1 are defined in Table 10.5.

10.2 Properties

10.2.1 Chemistry

10.2.1.1 General properties

The structure of folic acid is given in Figure 10.2. Pteroic acid, 4-[(pteridin-6-ylmethyl)amino] benzoic acid, is the parent compound.[46] Folate refers to the large group of heterocyclic compounds based on the pteroic acid structure conjugated with one or more L-glutamates linked through the γ-carboxyl of the amino acid. Folic acid contains one glutamic acid residue with the accepted name "pteroylglutamic acid." Folic acid is not found in nature; however, it is the common, more stable, synthetic form used for food fortification and for formulation

Table 10.3 Folate Content of Various Foods[a]

Description	NDB no.	µg 100 g^{-1}	Description	NDB no.	µg 100 g^{-1}
Cereals ready-to-eat, GENERAL MILLS, Whole Grain TOTAL	08077	2690	Spaghetti, cooked, enriched, without added salt	20121	119
Cereals ready-to-eat, QUAKER, CAP'N CRUNCH	08010	2633	Rice, white, long-grain, precooked or instant, enriched, prepared	20049	118
Cereals ready-to-eat, GENERAL MILLS, TOTAL Corn Flakes	08246	2253	Turnip greens, cooked, boiled, drained, without salt	11569	118
Cereals ready-to-eat, KELLOGG, KELLOGG'S FROSTED FLAKES	08069	542	Cereals, CREAM OF WHEAT, mix'n eat, plain, prepared with water	08109	116
Rice, white, long-grain, parboiled, enriched, dry	20046	431	Soybeans, green, cooked, boiled, drained, without salt	11451	111
Rice, white, long-grain, regular, raw, enriched	20044	387	Broccoli, cooked, boiled, drained, without salt	11091	108
Cornmeal, self-rising, degermed, enriched, yellow	20025	375	Broccoli, cooked, boiled, drained, without salt	11091	108
Cornmeal, de germed, enriched, yellow	20022	363	Noodles, egg, cooked, enriched	20110	104
Turkey, all classes, giblets, cooked, simmered, some giblet fat	05172	335	Brussels sprouts, frozen, cooked, boiled, drained, without salt	11101	101
Wheat flour, white, all-purpose, self-rising, enriched	20082	307	Spinach, canned, drained solids	11461	98
Wheat flour, white, all-purpose, enriched, bleached	20081	291	Rice, white, long-grain, regular, cooked	20045	97
Wheat flour, white, bread, enriched	20083	288	Collards, cooked, boiled, drained, without salt	11162	93
Cereals ready-to-eat, KELLOGG, KELLOGG'S RAISIN BRAN	08060	275	Lima beans, large, mature seeds, cooked, boiled without salt	16072	83
Beef, variety meats and by-products, liver, cooked, pan-fried	13327	260	Cereals, CREAM OF WHEAT, quick, cooked with water, without salt	08105	74
Cowpeas, common (blackeyes, crowder, southern), mature seeds, cooked, boiled, without salt	16063	208	Chickpeas (garbanzo beans, bengal gram), mature seeds, canned	16058	67
Lentils, mature seeds, cooked, boiled, without salt	16070	181	Fast foods, hotdog, plain	21118	62
Beans, pinto, mature seeds, cooked, boiled, without salt	16043	172	Peas, green, frozen, cooked, boiled, drained, without salt	11313	59
Orange juice, frozen concentrate, unsweetened, undiluted	09214	155	Fast foods, hamburger, regular, double patty, with condiments	21111	53
Okra, frozen, cooked, boiled, drained, without salt	11281	146	Corn, sweet, yellow, canned, cream style, regular pack	11174	45
Spinach, cooked, boiled, drained, without salt	11458	146	Orange juice, frozen concentrate, unsweetened, diluted with 3 volume water	09215	44
Asparagus, frozen, cooked, boiled, drained, without salt	11019	135	Lettuce, iceberg (includes crisphead types), raw	11252	29
Beans, kidney, red, mature seeds, cooked, boiled, without salt	16033	130	Tomato juice, canned, with salt added	11540	20
Rice, white, long-grain, parboiled, enriched, cooked	20047	127	Eggs, scrambled, frozen mixture	43285	17
Spaghetti, cooked, enriched, without added salt	20121	123	Yogurt, fruit variety, nonfat	43261	9
Macaroni, cooked, enriched	20100	123	Ice creams, vanilla, rich	19089	8
Spinach, frozen, chopped or leaf, cooked, boiled, drained, without salt	11464	121	Milk, whole, 3.25% milkfat	01077	5

[a] Data from USDA *National Nutrient Database for Standard Reference, Release* 19.

Source: United States Department of Agriculture, Agricultural Research Service, 2006, USDA Nutrient Database for Standard Reference, Release 19, Nutrient Data Laboratory Home Page, http://www.nal.usda.gov/fnic/foodcomp, Riverdale, MD, Nutrient Data Laboratory, USDA.

Table 10.4 Dietary Reference Intakes and Tolerable Upper Intake Levels for Folate

Life stage	DRI ($\mu g\ d^{-1}$)	UL ($\mu g\ d^{-1}$)
Infants (months)		
0–6	65	[a]
7–12	80	[a]
Children (years)		
1–3	**150**	300
4–8	**200**	400
Males (years)		
9–13	**300**	600
14–18	**400**	800
19–30	**400**	1000
3–50	**400**	1000
51–70	**400**	1000
>70	**400**	1000
Females (years)		
9–13	**300**	600
14–18	**400**	800
19–30	**400**	1000
31–50	**400**	1000
51–70	**400**	1000
>70	**400**	1000
Pregnancy (years)		
≤18	**600**	800
19–30	**600**	1000
31–50	**600**	1000
Lactation (years)		
≤18	**500**	800
19–30	**500**	1000
31–50	**500**	1000

Bold type: Recommended Dietary Allowance: ordinary type: adequate intake (AI).

[a] Not possible to establish; source of intake should be formula and food only.

Source: Food and Nutritional Board, Institute of Medicine, *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B-6, Folate, Vitamin B-12, Pantothenic Acid, Biotin and Choline*, National Academy of Sciences Press, Washington, DC, 1998, chap. 8.

of pharmaceuticals. The United States Pharmacopeial Convention (USP) Reference Standard is folic acid. Folic acid must be reduced to H_2 folate or tetrahydrofolate (H_4 folate). Tetrahydrofolate is the active coenzyme form of the vitamin. Folate structures and nomenclature are quite complicated owing to the number and diversity of biologically active forms. In general, structural variations encompass changes in the oxidation state of the pteridine ring structure, the one-carbon moiety carried by the specific folate, and the number of conjugated glutamate residues on the specific folate. For clarification, the International Union of Pure and Applied Chemistry–International Union of Biochemistry (IUPAC–IUB) nomenclature rules are briefly summarized as follows.[46]

1. Pteroic acid conjugated with one or more L-glutamate units are named pteroylglutamate, pteroyldiglutamate, and so forth. The name "pteroylmonoglutamate" should not be used (IUPAC-IUB Recommendation 2.3).

Table 10.5 Enzymes of Folate Metabolism

	Enzyme	EC number
[a]1.	γ-Glutamyl hydrolase	EC 3.4.22.12 (Brush border)
2.	Dihydrofolate reductase	EC 1.5.1.3
3.	Folypoly-glutamate synthetase	EC 6.3.2.17
4.	Serine hydroxymethyl transferase	EC 2.1.2.1
5.	Methylenetetrahydrofolate reductase	EC 1.7.99.5
6.	γ-Glutamyl hydrolase	EC 3.4.22.12 (lysosomal)
7.	Cobalamin-dependent methionine synthase	EC 2.1.1.13
8.	Glycine cleavage enzyme system	EC 1.4.4.2 EC 2.1.2.10
9.	Glutamate formiminotransferase	EC 2.1.2.5
10.	Formiminotetrahydrofolate cyclodeaminase	EC 4.3.1.4
11.	Methylenetetrahydrofolate dehydrogenase	EC 1.5.1.5
12.	5,10-Methenyltetrahydrofolate cyclohydrolase	EC 3.5.4.9
13.	10-Formyltetrahydrofolate synthetase	EC 6.3.4.3
14.	Thymidylate synthase	EC 2.1.1.45
15.	10-Formyltetrahydrofolate dehydrogenase	EC 1.5.1.6
16.	Glycinamide ribonucleotide transformylase (GAR)	EC 2.1.2.2
17.	Phosphoribosylamino-imidazole carboxamide transformylase	EC 2.1.2.3
18.	5-Formyltetrahydrofolate cycloligase	EC 6.3.3.2
19.	Folate/MTX transport mechanism	
20.	Glycine methyl transferase	EC 2.1.1.20

[a] Numbers refer to enzymes in Figure 10.1.

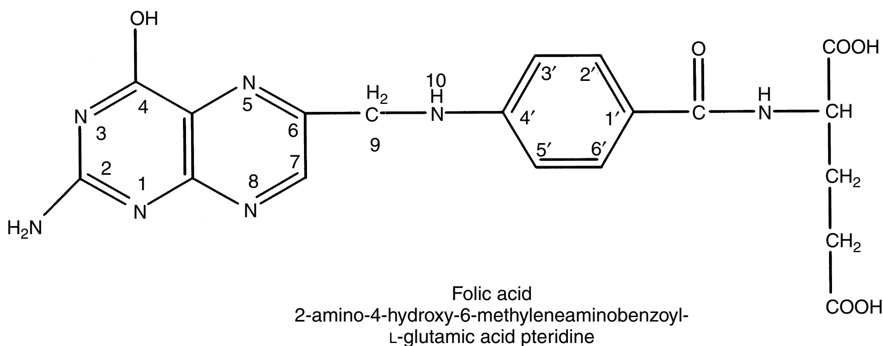

Figure 10.2 The structure of folic acid.

2. Folate and folic acid are the preferred synonyms for pteroylglutamate and pteroylglutamic acid, respectively (IUPAC-IUB Recommendation 2.4).
3. "Folate" may be used to designate any members of the family of pteroylglutamates or mixtures with various levels of reduction of the pteridine ring, single-C substitutions, and numbers of conjugated glutamate residues (IUPAC-IUB Recommendation 2.5).
4. Reduced compounds are indicated by the prefixes dihydro-, tetrahydro-, and so forth, with numerals indicating the positions of the additional hydrogens. Tetrahydrofolate is assumed to be substituted in the 5, 6, 7, and 8 positions, and H_2 folate is assumed to be substituted in the 7 and 8 positions unless otherwise indicated (IUPAC-IUB Recommendation 2.6).
5. Substituent groups are indicated by prefixes together with the locations of the positions substituted. Substituent prefixes indicate that the substituent replaces one hydrogen

atom in the parent structure in the case of formyl, methyl, or formimino, or two hydrogens in the case of methylene and methenyl (IUPAC-IUB Recommendation 2.7).

6. Rules for use of symbols and abbreviations include the following:
   a. Folate is preferred to folic acid and should not be abbreviated.
   b. Pteroate, pteroyl, or pteroic acid is indicated by the symbol "Pte."
   c. Pteroylglutamates and the corresponding acids are indicated by the symbols PteGlu, $PteGlu_2$, $PteGlu_3$, and so forth. The subscript indicates the number of glutamate units.
   d. Reduced states are indicated by H_2 or H_4 in front of the main symbol. H_4 folate and H_2 folate are preferred abbreviations. TH and DH should not be used.
   e. Substituents are indicated as follows:

Substituent	Symbol	Formula
Formimino	NHCH–	HN=CH–
Formyl	HCO–	O=CH–
Methyl	CH_3–	CH_3–
Methylene	–CH_2–	–CH_2–
Methenyl	–CH^+	–CH= as a component of $[>N–CH=N^+< \leftrightarrow >N^+ = CH–N<]$

The above rules regarding symbols and abbreviations are found in IUPAC–IUB Recommendation 2.8. Names of substituted folates, abbreviations, and substituent positions are provided in Table 10.6. Structural relationships are shown in Figure 10.3.

Physical and chemical properties of folic acid and reduced folates were reviewed in detail by Temple and Montgomery[47] and Gregory.[48] Solubility characteristics summarized by Gregory[48] include the following:

1. Folates exhibit minimum solubility in mildly acidic solvents (pH 2–4) where monocationic and neutral forms predominate.
2. Solubility increases in proportion to the pH above this range as anionic species increase in concentration.
3. Solubility increases at very low pH in strong acid solutions. At such low pH levels, highly cationic species exist.
4. Pteroic acid is less soluble than folic acid over most of the pH scale. The solubility of folic acid is influenced by the polar hydrophilic character of the a-carboxyl group of glutamic acid.
5. At neutral to alkaline pH levels, polyglutamyl folates are more anionic than folic acid because of the presence of additional ionizable a-carboxyl groups.

Table 10.6 Substituent Groups and Their Positions in Folic Acid Enzymes

Name	Abbreviation	Position	
		N-5	N-10
Pteroylglutamic acid	Folic acid	—	–H
7,8-Dihydrofolate	H_2 folate	–H	–H
5-Methyl-5,6-dihydrofolate		–CH_3	–H
5,6,7,8-Tetrahydrofolate	H_4 folate	–H	–H
5-Methyltetrahydrofolate	5-CH_3–H_4 folate	–CH_3	–H
5-Formyltetrahydrofolic acid	5-CHO–H_4 folate	–CHO	–H
10-Formyltetrahydrofolate	10-CHO–H_4 folate	–H	–CHO
5,10-Methenyltetrahydrofolate	5,10-CH^+=H_4 folate	=CH- ± bridge	
5,10-Methylenetetrahydrofolate	5,10-CH_2H_4 folate	–CH_2- bridge	
5-Formiminotetrahydrofolate	5-CHNH–H_4 folate	–CHNH	–H

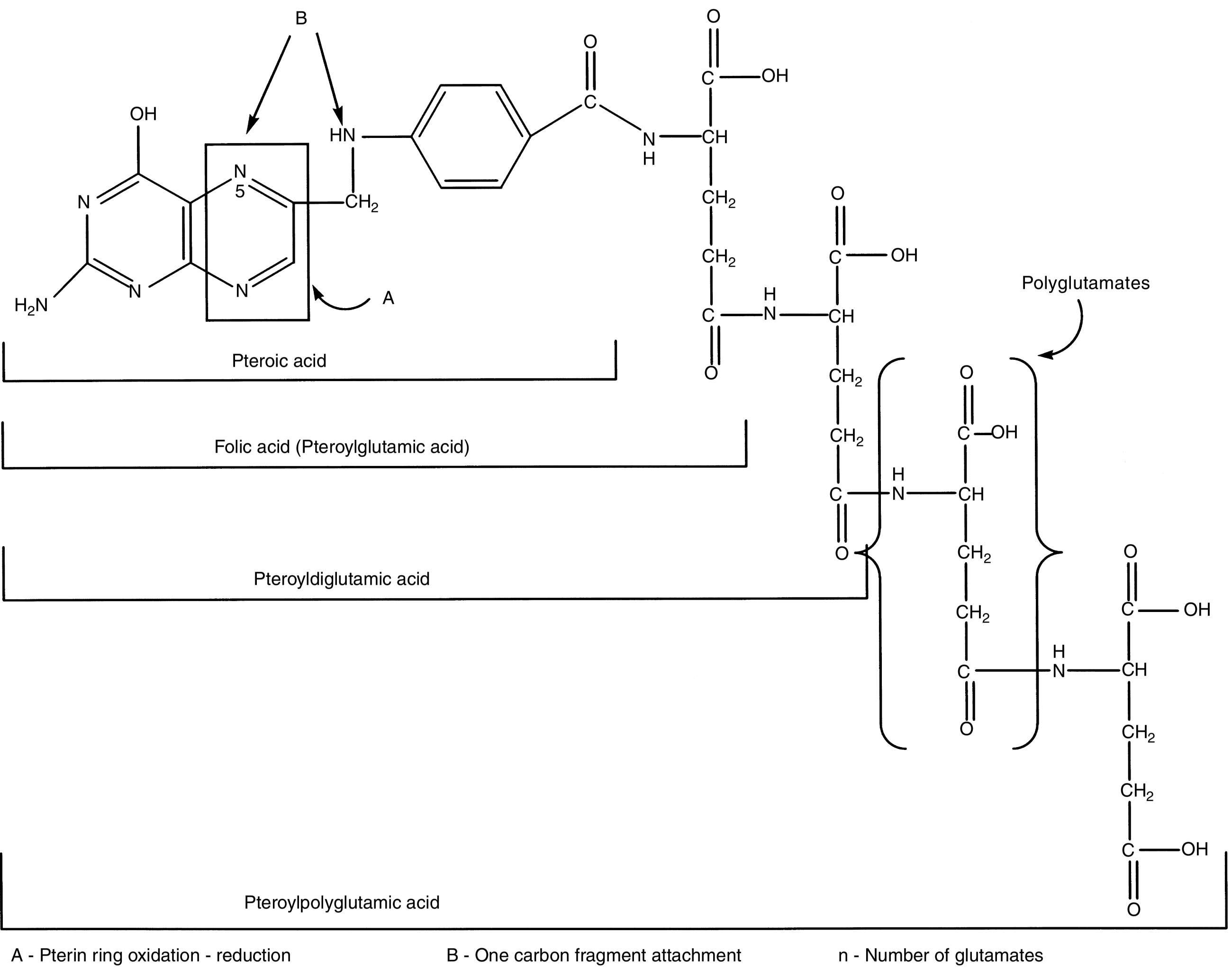

Figure 10.3 Structural relationships of the folates.

6. Long-chain polyglutamyl folates are more hydrophobic than short-chain polyglutamyl folates at low pH levels where the a-carboxyl groups are predominantly protonated.

10.2.1.2 Spectral properties

Absorption and fluorescence properties of folic acid and commonly occurring derivatives are given in Table 10.7. For more detailed spectral information, the reader is referred to extensive tabular information provided by Temple and Montgomery.[47] Descriptive ultraviolet (UV) spectra and fluorescence excitation and emission spectra were provided by Ball.[49] The spectra of 5-methyltetrahydrofolate is shown in Figure 10.4. Folates show characteristic UV absorption spectra usually with three absorbance maxima, such as 256, 283, and 368 nm.

A maxima attributable to the *p*-aminobenzoyl-glutamic acid (PABG) moiety exists from 270 to 280 nm.[49] H_4 folate exhibits a single maximum at 286 nm in 0.1 M phosphate buffer, pH 7.1.[49] Likewise, PABG strongly influences fluorescence properties of the folates. Reduced pterins without the *p*-aminobenzoyl moiety do not exhibit native fluorescence.[49] The most comprehensive study on fluorescence properties of the pteridines was published by Uyeda and Rabinowitz[52] and is often referenced as the single-most significant source on fluorescence properties of the folates. H_2 folate and H_4 folates, other than 5,10-methenyl-H_4 folate, fluoresce maximally at excitation 300–320 nm and emission 360–425 nm. Folate has an excitation maximum at 360–380 nm with emission maximum at 450–460 nm.[51,52] Fluorescence intensity is strongly influenced by pH and buffer composition. For reduced folates, fluorescence intensity increases as acidity of the solvent increases. For high-performance liquid chromatography (HPLC) determination by fluorescence, native fluorescence of H_4 folate, 5-methyl-H_4 folate, and 5-formyl-H_4 folate is sufficient at pH 2.3 to allow detection at low pmol detection limits. Fluorescence intensity of H_2-folate and 10-formyl-H_4 folate is not sufficient to allow quantitation, and folic acid does not fluoresce.[52,53] Procedures used to apply fluorescence to liquid chromatography (LC) quantitation of these analytes are discussed in Section 10.4. Fluorescence is considered the most specific and, in most cases, most sensitive detection mode for LC detection.[48,53]

10.2.1.3 Stability

Several excellent reviews exist that comprehensively cover the extensive information available on folate stability.[48–50,54,55] To call attention to specific stability properties of importance to the analyst, the following summary is provided along with primary and secondary literature references. The summary of stability characteristics is not meant to be all inclusive, but to provide a guide to analysts to be aware of the many factors that affect folate stability in biological systems and during analysis.

1. Loss of biological activity occurs through oxidative cleavage of the C-9-N-10 bond.
   a. *p*-Aminobenzoylglutamate is the major oxidation product at pH 4, 7, and 10. Pterin fragments include predominantly pterin at pH 4 and 6, and formylpterin at pH 7 and 10.[51,54,56]
   b. H_4 Folate is more susceptible to oxidation than folic acid.[48,54,57]
   c. Substitution at the *N*-5 and *N*-10 positions increases oxidative stability of reduced folates.[48,49,56]
   d. Reducing agents including ascorbic acid, 2-mercaptoethanol, and dithiothrietol stabilize folates.[48,49,54,55,57,58,59,60]
   e. Folic acid is more stable than naturally occurring folates at both ambient and elevated temperatures.[48,49,55,56]
   f. Folic acid is stable at 100°C when protected from light at pH 5.0–12.0.
   g. 5-Formyl–H_4 folate is stable at neutral pH. Under acidic conditions with heating, it is converted to 5,10-methenyl-H_4 folate.

Table 10.7 Physical Properties of Folates

Substance[a]	Molar mass	Formula	Absorbance[b]				Fluorescence[c]		
			λ max	$E^{1\%}_{1\text{cm}}$	ε × 10^{-3}	Solvent	Ex (nm)	Em (nm)	pH
Folic acid	441.40	$C_{19}H_{19}N_7O_6$	282	[611.7]	27.0	Phosphate buffer, pH 7.0	363	450–460	9
CAS No. 59-20-3			350	[158.6]	7.0	Phosphate buffer, pH 7.0			
4253			254	[566.4]	25.0	*M* NaOH, pH 13			
			282	[532.2]	23.8	0.1 *M* NaOH, pH 13			
			363	[197.1]	8.7	0.1 *M* NaOH, pH 13			
H_4 folate	445.44	$C_{19}H_{23}N_7O_6$	297	[606.1]	27.0	Phosphate buffer, pH 7.0	305–310	360	3
10-CHO–H_4 folate	473.45	$C_{20}H_{23}N_7O_7$	288	[384.4]	18.2	Phosphate buffer, pH 7.0	313	360	7
5-CHO–H_4 folate	473.45	$C_{20}H_{23}N_7O_7$	287	[665.3]	31.5	Phosphate buffer, pH 7.0	314	365	7
CAS No. 58-05-9			282	[688.5]	32.6	0.1 *M* NaOH, pH 13			
4254									
5-CH_3–H_4 folate	459.46	$C_{20}H_{25}N_7O_6$	290	[696.5]	32.0	Phosphate buffer, pH 7.0	—	—	—
5-CHNH–H_4 folate	472.46	$C_{20}H_{24}N_8O_6$	285	[749.3]	35.4	Phosphate buffer, pH 7.0	308	360	7
5,10-CH=H_4 folate	456.44	$C_{20}H_{22}N_7O_6$	352	[547.7]	25.0	Phosphate buffer, pH 7.0	370	470	4
5,10-CH_2–H_4 folate	457.45	$C_{20}H_{23}N_7O_6$	294	[699.5]	32.0	Phosphate buffer, pH 7.2	—	—	—

[a] Common or generic name; CAS No: Chemical Abstract Service number, bold print designates the Merck Index monograph number.
[b] Values in brackets are calculated from corresponding $E^{1\%}_{1\text{cm}}$ values.
[c] pH of optimum fluorescence.

Sources: Friedrich, W., Folic acid and unconjugated pteridines, In *Vitamins*, Walter de Gruyter, Berlin, 1988, chap. 10.
Temple, C., Jr. and Montgomery, J. A., Chemical and physical properties of folic acid and reduced derivatives, In *Folates and Pterins*, vol. 1, Blakley, R. L. and Benkovic, S. J., eds., John Wiley & Sons, New York, 1984, chap. 2.
Ball, G. F. M., Folate, In *Water-Soluble Vitamin Assays in Human Nutrition*, Chapman & Hall, New York, 1994, chap. 2.
Budavari, S., *The Merck Index*, 13th ed., Merck and Company, Whitehouse Station, NJ, 2001, p. 748.

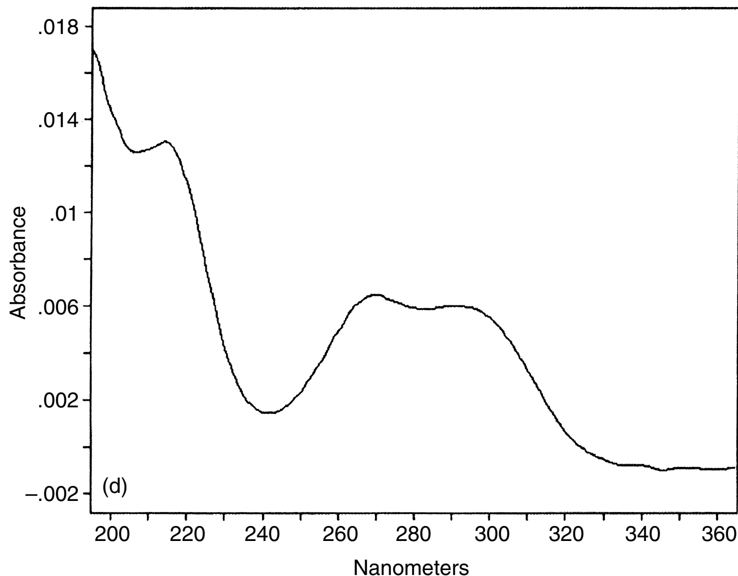

Figure 10.4 UV/Vis spectra 5-CH_3-H_4 folate from 200–360 nm. (Reproduced from Phillips, K. M., Wunderlich, K. M., Holden, J. M., Exler, J., Gebhardt, S. E., Haytowitz, D. B., Beecher, G. R., and Doherty, R. F., *Food Chem.*, 92, 587, 2005. With permission.)

2. The number of glutamate residues attached to the folate does not influence stability.[49,54]
3. Folates are cleaved through photochemical and reductive mechanisms.[48,49,61,62]
4. Ultraviolet radiation cleaves folic acid into *p*-aminobenzoyl-L-glutamic acid and 6-formyl pterin. The 6-formyl pterin can be further degraded into pterin-6-carboxylic acid.[63]
5. Folate is subject to food processing and storage losses; however, the losses are variable owing to the variations in food matrices, oxygen availability, chemical environment, extent of heating, and forms of folate in the food.[64]
   a. Folates, being water soluble, are subject to large leaching losses. Canning liquid can contain a significant proportion of the folate originally present in the raw product.[64,65]
   b. Presence of reducing agents (ascorbic acid) in food can increase folate retention during thermal processing.[54,66,67,68]
   c. Presence of metals (Fe^{2+}) can increase folate loss.[54,69]
   d. Common food additives including sodium nitrite used in some cured products cause folate destruction.[48,70]
   e. Kinetics of thermal loss generally follow first-order rates and depend on oxygen concentration (pseudo-first-order rate).[54,55,71]
   f. As dissolved or headspace oxygen levels decrease, folate stability increases.[21,37,48,49,54,72]
   g. Folate is quite stable in dry products in the absence of light and oxygen. Of significance to the addition of folic acid to cereal products, the vitamin is stable in flour during storage and subject to only small losses during baking.[73,74,75,76]
   h. Folic acid is used for fortification. Its stability is greater than naturally occurring folates in most foods.
   i. Stability of 5-methyltetrahydrofolate is excellent in frozen fresh vegetables and fruits.[77,78]

Considerable data are available on the stability of 5-methyl-H_4 folate in fluid milk. Milk provides an ideal product for thermal processing studies because 90%–95% of the folate in milk is 5-methyl-H_4 folate.[79] Its stability in milk has been well defined.[66,67,80–82] Loss during UHT pasteurization is usually less than 20%. Recent ultra high temperature (UHT) thermal processing studies in model systems showed 5-methyl-H_4 folate degradation as second order in the presence of oxygen.[82] Degassing of liquid foods before thermal processing to reduce dissolved oxygen levels greatly improves folate retention. Folate was stable in refrigerated serum for up to 7 days.[83] In tablets, folic acid is stable with expiration times increasing in coated tablets.

10.2.1.4 Bioavailability

In-depth discussion of the bioavailability of folate to the human is much beyond the scope of this text. An excellent summary of factors influencing vitamin bioavailability is given in Table 10.8.[83] Most, if not all, of these factors apply to folate. Significant to absorption, polyglutamylfolates are hydrolyzed to monoglutamylfolates in the brush border (Figure 10.1).[94–96] The enzyme(s), γ-glutamyl hydrolase or human conjugase, can be inhibited by food substances.[97] The polyglutamate chain is generally considered to reduce bioavailability.[98] Processing and cooking effects on conjugase inhibitors are largely unknown. Conversely, folate-binding protein from milk may increase folate absorption by protecting dietary folates from uptake by bacteria in the gut, thus increasing absorption in the small intestine.[99] Other studies, however, have contradicted early suggestions regarding the role that dietary binding proteins might play in human or animal absorption of folates.[100] Recent

Table 10.8 Factors Influencing Bioavailability of Vitamins[a]

Factor	Biological or physical variable
Extrinsic	
Chemical form of vitamin	Solubility or dispersibility
	Difference in rates of absorption, transport, and metabolism
	Potential variation in tissue uptake, renal, and biliary excretion
Concentration of vitamin	Concentration dependence of intestinal absorption
	Solubility of vitamin
Physical form of vitamin	Formulation variables (coatings, counter-ions, emulsifiers, etc.)
	Physical interactions with other food components
Composition of diet/food	Digestibility (protein, fat, fiber)
	Influence on intestinal digestion processes
	Influence on intestinal emulsification and absorption of lipids and fat-soluble vitamins
	Influence on intestinal absorption processes
	Intestinal transit time
	Influence of diet on intestinal microflora
Nonfood antagonists	Impaired absorption or metabolism caused by pharmaceuticals or alcohol
Intrinsic	
Age of individual	Developmental changes in digestive function
	Age-induced changes in gastrointestinal function
Species	Human versus animal model
	Is animal model appropriate?
Health	Digestive function (e.g., secretion of gastric HCl and pepsin, pancreatic enzymes, and bile acids)
	Nutritional status

[a] Table reproduced with permission from Reference 83.

studies have shown that inclusion of bovine milk in the diet enhances folate bioavailability. However, the mechanism of action was not defined.[101] Research by Verwei et al.[102] found that the addition of folate-binding protein to milk decreased the bioavailability of folic acid and 5-CH3–H4 folate from fortified milk. However, the overall effect of folate-binding proteins in a person's everyday diet remains unclear.

Food component interactions with dietary folates may act to either increase or decrease efficiency of folate absorption. Excellent studies on fiber effects on folate bioavailability indicate lack of binding, but a potential for physical interferences with absorption mechanisms at the surface of the intestinal mucosa.[48,103–106] Other dietary interactions include effects of foods on intestinal pH with potential modification of conjugase activity, presence of folate antagonists, intestinal changes influenced by dietary factors (alcoholism), chelation, and factors that influence the rate of gastric emptying. In spite of the large amount of information available on folate bioavailability, knowledge of this important part of folate nutrition is not complete. Many informative reviews exist for those requiring more complete information on the bioavailability of folic acid and folate.[48,55,95,96,84,107–116]

10.3 The μg dietary folate equivalents

Studies by Gregory,[84] Pfeiffer et al.,[85] Cuskelly et al.,[86] and Wei et al.[87] explored differences in bioavailability of folic acid used in supplements and fortification compared to folate from food sources. These studies and earlier studies by Sauberlich et al.[88] were used by the Institute of Medicine[9] to recommend use of the μg DFE to adjust for differences in bioavailability of folic acid and food folates. Values of bioavailability used to establish the μg DFE were folic acid with food—85% bioavailable, folic acid from supplements without food—100% bioavailable, and food folate—50% bioavailable. When a mixture of folic acid and folate from food is injected, folic acid is 85/50 or 1.7 times more bioavailable compared to the food folate. μg DFEs in this situation are calculated by the formula:[89]

$$\mu g\ DFE = \mu g\ food\ folate + 1.7 \times \mu g\ folic\ acid$$

According to Gregory,[84] supplements of folic acid consumed on an empty stomach are 100% bioavailable. Hence, compared with food folate, only half as much folic acid is necessary if taken on an empty stomach. Therefore, the following relationships are valid:

1 μg DFE provided = 1 μg food folate = 0.5 μg folic acid taken on an empty stomach
= 0.6 μg folic acid taken with meals.

Use of μg DFE has been fully discussed by Bailey,[90,91] Bailey and Gregory,[92] and Sutor and Bailey.[93]

10.4 Methods

Methods for folate analysis include bioassays, microbiological, LC, ligand-binding, and radioimmunoassay (RIA). In the last 5 years, LC combined with mass spectrometry (LC-MS) methods have added another level of specificity to LC methods developed in the 1990s and offer the best approach to precisely define specific folate profiles in biological samples. Excellent reviews are available that cover the many aspects of folic acid—folate analysis.[48,49,117,118] Keagy[119] reviewed chick and rat bioassays. Folate analysis is complicated by the multiple metabolic forms present in biological samples, variable γ-glutamyl polymer lengths of 2–12, and instability, which must be of primary concern to all analysts involved with folate assay. Many established and/or regulatory procedures are available in handbooks. These methods are summarized in Table 10.9; however, folate analysis is rapidly advancing and newer LC or LC-MS methods offer many advantages.

Table 10.9 Regulatory and Handbook Methods for Analysis of Folate and Folic Acid

Source	Form	Methods and application	Approach	Most current cross-reference
***U.S. Pharmacopeia National Formulary*, 2006, USP 29/NF 24, Dietary Supplements Official Monographs**[120]				
1. Pages 2392, 2395, 2398, 2416–2417	Pteroylglutamic acid	Folic acid in oil- and water-soluble capsules/tablets w/wo minerals	Method 1—LC 280 nm Method 2—LC 270 nm	None
2. Pages 2427, 2430, 2431, 2436	Pteroylglutamic acid	Folic acid in water-soluble vitamin capsules/tablets w/wo minerals	Method 1—LC 280 nm Method 2—LC 270 nm	None
3. Pages 965–966	Pteroylglutamic acid	Folic acid (NLT 97.0%, NMT 102.0%)	LC 280 nm	None
4. Pages 966–967	Pteroylglutamic acid	Folic acid injection/tablets	LC 254 nm	None
***British Pharmacopoeia*, 2007**[121]				
1. Pages 922–923	Pteroylglutamic acid	Folic acid	Spectrophotometric 550 nm	None
2. Page 2603	Pteroylglutamic acid	Folic acid tablets	LC 283 nm	None
3. Pages 338–340	Calcium folinate	Calcium folinate	LC 280 nm	None
4. Pages 2380–2381	Calcium folinate	Calcium folinate injection	LC 280 nm	None
5. Page 2381	Calcium folinate	Calcium folinate for injection	LC 280 nm	None
6. Pages 2381–2382	Calcium folinate	Calcium folinate tablets	LC 280 nm	None
7. Pages 2576–2577	Folic acid	Ferrous fumarate and folic acid tablets	LC 277 nm	None
***AOAC Official Methods of Analysis*, 18th ed., 2005**[122]				
1. 45.2.03	Pteroylglutamic acid	AOAC Official Method 944.12, Folic Acid (Pteroylglutamic acid) in Vitamin Preparations	Microbiological	
2. 50.1.21	Pteroylglutamic acid and naturally occurring forms	AOAC Official Method 992.05 Folic Acid (Pteroylglutamic acid) in Infant Formula	Microbiological	*J. AOAC Int.*, 76, 399, 1993[123]
3. 45.2.09	Total folates	Cereals and Cereal Foods	Microbiological trienzyme	*J. AOAC Int.*, 88, 5, 2005[124]
***Food Chemicals Codex*, 5th ed., 2004**[125]				
1. Page 180	Folic acid	Folic acid (NLT 95.0%, NMT 102.0%)	LC 254 nm	None
***American Association of Cereal Chemists Approved Methods*, 10th ed., vol. 2, 2000**[126]				
1. AACC 86-47		Total folate	Microbiological trienzyme	*Cereal Food World*, 46, 216, 2001[127]

10.4.1 Microbiological

10.4.1.1 Folate assay organisms

Three bacteria and the protozoan *Tetrahymena pyriformis* have been used for folate assay. The bacteria, *L. casei* ssp. *rhamnosus* ATCC No. 7469 (formerly *L. casei*), *Enterococcus hirae* ATCC No. 8043 (formerly *S. faecalis*), and *Pediococcus acidilactici*, ATCC No. 8081 (formerly *P. cerevisiae*) have different responses to the folates available in biological matrices. Their responses and the limited information available on the response of *Tetrahymena pyriformis* are shown in Table 10.10. *L. casei* ssp. *rhamnosus* is the most commonly used and most accepted organism for folate analysis of natural products. It responds to natural folate forms present in biologicals and does not respond to pteroic acid—a common folate degradation product. *Enteroeoecus hirae* lacks specificity, in that it does not respond to 5-methyl-H_4 folate, the most common folate present in milk, other foods, tissue, and serum, and does respond to pteroic acid.[128] None of the organisms efficiently responds to γ-glutamyl folate with greater than three glutamic acid residues. *L. casei* ssp. *rhamnosus* has greater capacity for response to the γ-glutamyl folate polymers compared to the other assay organisms; however, its response is limited to no greater than three glutamates with much lower response to higher polymeric folates. The response of *L. casei* ssp. *rhamnosus* to pteGlu3 has been shown to be less than that to folic acid and pteGlu2.[129] Lack of response to the higher γ-glutamyl folate polymers requires γ-glutamyl hydrolase (conjugase) treatment during the extraction phase of the assay (Section 10.5.1.2) for each of the bacteria. *P. acidilactici* has the most limited response and can grow on only mono-, di-, and tri-glutamates of 5- or 10-$HCO–H_4$ folate. It does not respond to methyl-substituted folates.[49]

Even though *L. casei* ssp. *rhamnosus* is considered to be the best available bacteria for folate assay of food and other biologicals, its ability to respond equally on a molar basis to metabolically active folates has been questioned.[129] Goli and Vanderslice[129] reported significant growth response differences to various folates. Under the conditions of their study, the relative growth response to H_2 folate and H_4 folate were only 29% and 15% at higher vitamin concentration levels compared to folic acid. Response to 5-$CH_3–H_4$ folate, 5-$CHO–H_4$ folate,

Table 10.10 Responses Induced by Folate Derivatives Relative to Folic Acid

Folate	L. *rhamnosus*	E. *hirae*	P. *acidilactici*	T. *pyriformis*
Folic acid	+	+	—	+
H_2 folate	+	+	—	*[a]
H_4 folate	+	+	+	+
5-$HCO–H_4$ folate	+	+	+	+
10-$HCO–H_2$ folate	+	+	—	*
10-$HCO–H_4$ folate	+	+	+	*
5-$CH_3–H_4$ folate	+	—	—	—
5,10-$CH^+=H_4$ folate	+	+	—	*
5-$CHNH–H_4$ folate	+	+	+	*
$PteGlu_2$	+ (100%)	+	—	+
$PteGlu_3$	+ < (100%)	—	—	+
$PteGlu_4$	+ (65%)	—	—	*
$PteGlu_5$	+ (20%)	—	—	*
$PteGlu_6$	+ (3.6%)	—	—	*
$PteGlu_7$	+ (2.4%)	—	—	*
Pteroic acid	—	+	—	—

[a] Asterisk indicates that data is unavailable.

Source: Voigt, M. N. and Eitenmiller, R. R., Comparative review of the thiochrome microbial and protozoan analyses of B-vitamins, *J. Food Prot.*, 41, 730, 1978.

and 10-HCO-H_4 folate were 83%, 115%, and 95%, respectively, compared to folic acid. Reports of growth variations by *L. casei* ssp. *rhamnosus* are not common. Newman and Tsai[130] reported small differences between folic acid, 5-CH_3–H_4 folate, and 5-HCO–H_4 folate using a microtiter plate technique. Since standard curves are, in most circumstances, established with folic acid when the assay organism is *L. casei* ssp. *rhamnosus*, unequal growth response to the naturally occurring forms in a biological sample could significantly affect accuracy. Therefore, all analysts using the *L. casei* ssp. *rhamnosus* assay should determine growth response under their laboratory conditions to folic acid and major metabolic forms. Unequal response adds uncertainty to quantitative studies unless response factors are unequivocally proven.[117]

10.4.1.2 Extraction procedures

10.4.1.2.1 General requirements. Many procedures exist in the literature for extraction of folates for microbiological analysis, and procedures are still being refined. Traditional procedures include homogenization of the sample in buffers containing reducing agents such as ascorbic acid, 2-mercaptoethanol, and dithiothreitol to prevent oxidation. Protection from light and air help to maintain natural folates in their native states. Following homogenization, the sample homogenate is usually heated briefly at 100°C. The initial steps are designed to free the folate from the sample matrix, deproteinate the extract, and protect the native state folates. In addition, higher fat samples should be defatted to prevent fatty acid stimulation of the growth response of *L. casei* ssp. *rhamnosus*.[131] Plasma analysis follows similar protocols used for food and other biologicals. Simple dissolution in buffer containing reducing agents is sufficient for pharmaceuticals containing only folic acid.

Biological samples must be hydrolyzed with γ-glutamyl hydrolase (conjugase) to cleave poly-γ-glutamyl folates to the triglutamate level or lower for growth of *L. casei* ssp. *rhamnosus* and to lower degrees of polymerization for *E. hirae*. Conjugases from several sources are usable. Chicken pancreas conjugase is specified in Association of Official Analytical Chemists (AOAC) International methods.[122] Crude, dried chicken pancreas is available commercially and provides a convenient source for the enzyme. Other common sources are hog kidney, rat, and human plasma. Hog kidney, rat, and human plasma conjugase produce monoglutamyl folates and, therefore, serve as the hydrolases for LC methods requiring complete deconjugation. Keagy[119] provides properties of several conjugases used by various investigators (Table 10.11). Unless organisms other than *L. casie* ssp. *rhamnosus* are used

Table 10.11 Properties of γ-Glutamyl Hydrolases Used for Folate Assay

Source	pH optimum	Glutamate residues in product
Chicken pancreas	7.8	2
Hog kidney	4.5	1
Rat pancreas	5.5–6.0	1
Human jejunum	4.5	1
Human plasma	6.5	1
Liver	4.5	1
Cabbage	5.0	1 or 2
Cabbage	8.0	2

Source: Keagy, P.M., Method, of Vitamin Assay, Augestin, J., Klein, B.P., Becker, D.A., and Venugopal, P.B., eds., John Wiley & Sons, New York, Chap. 81.

as the assay organism or LC analysis is to be completed that requires only monoglutamates in the digest, chicken pancreas provides a suitable and reliable conjugase source. Properly handled, residual folate from the chicken pancreas preparations will be low and will not interfere with the assay. Most commercial acetone-washed chicken pancreas preparations can be used with minimal cleanup. However, a method provided by Keagy[119] can be used to produce a low folate chicken pancreas preparation. The steps are as follows:

1. Suspend 10 g of dessicated chicken pancreas (Difco) in 300 mL 0.1 M phosphate buffer, pH 7.0. Stir 1 h at room temperature.
2. Incubate overnight at 30°C under toluene, centrifuge.
3. Mix the supernatant with an equal volume of 0.1 M tricalcium phosphate gel suspension (Sigma) for 30 min. Cool in an ice bath. Centrifuge cold.
4. Cool the upper layer below 5°C and slowly add an equal volume of ice cold ethanol with vigorous stirring. Refrigerate overnight.
5. Centrifuge. Dissolve precipitate in 100 mL of 0.1 M phosphate buffer, pH 7.0, by stirring. Container should be kept cold in an ice bath.
6. Centrifuge. Mix supernatant with 10% by weight of Dowex 1 × 8 (chloride) for 1 h at 0°C.
7. Remove Dowex by centrifugation and filter the supernatant through gauze.
8. Store frozen in volumes required for batch usage, depending on laboratory requirements.

Goli and Vanderslice[132] reported that *L. casei* ssp. *rhamnosus* response to di- and triglutamates was 90% and 60%, respectively, compared to the response to folic acid. Use of plasma conjugase at pH 4.5 produces monoglutamates, but significant folate degradation. The authors recommend pH 6.0 for use of plasma conjugase. Chicken pancreas conjugase yields a mixture of mono- and diglutamate folates at pH 6.0 and 7.0. The authors cautioned that a commonly used hog kidney conjugase digestion at pH 4.9 might lead to substantial folate degradation. Conversely, use of 0.05 M sodium acetate buffer, with 1% ascorbate was reported to give greater stability during extraction than at pH 7.0.[48,53] Gregory[48] concluded that mildly acidic buffers containing ascorbate are generally useful for folate extraction from most matrices.

10.4.1.2.2 Buffers and antioxidants. Many different buffer systems and stabilizing antioxidants have been used to extract folate from biological samples. Recent work is beginning to give a clearer picture of required parameters for optimal extraction. Buffer systems provide optimal pH conditions for enzymatic deconjugation in later extraction steps.[48] Phosphate buffers between pH 6.1 and 7.2 are used with chicken pancreas and hog kidney conjugase. Although the pH optimum of hog kidney conjugase is 4.5, use of buffers near pH 6.0 gives sufficient deconjugation, and avoids reported loss of folate at lower pH levels.[59] At pH 4.9, folate is stable at 100°C for 60 min if 1.0% ascorbate is present.[48] Acetate buffer has been used in conjunction with plasma conjugase for both blood and food analysis; however, acetate inhibits this conjugase.[59,132]

Phosphate buffer, pH 6.0, containing 52 mM (1%) ascorbic acid/ascorbate and 0.1% 2-mercaptoethanol is an excellent extractant for foods before HPLC analysis. This buffer system could be easily incorporated into microbiological assay protocols. The addition of the 2-mercaptoethanol produced better folate stability than use of only ascorbic acid. As discussed in Section 10.5.1.2.1, use of 0.05 M sodium acetate buffer, pH 4.9, with 1% ascorbate stabilizes folates during extraction. Acidic systems increase the stability of ascorbic acid, and are thought to eliminate degradation products from the extraction solution that can interact with the folate.[133]

Studies by Wilson and Horne[133,134] led to the use of 50 mM *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid (HEPES), 50 mM 2-(*N*-cyclohexylamino) ethanesulfonic acid

(CHES) containing 100 mM sodium ascorbate and 200 mM 2-mercaptoethanol, pH 7.85, for folate extraction from biological samples. The buffer is now commonly referred to as the Wilson and Horne buffer. The buffer system avoids folate interconversions attributed to ascorbate degradation products. Gregory et al.[135] evaluated the Wilson and Horne buffer (HEPES/CHES), pH 7.85; acetate buffer with 1% ascorbate, pH 4.9, and phosphate buffer with 1% ascorbate, pH 7.0, and found that the Wilson and Horne buffer was superior for folate extraction. Since this work, the Wilson and Horne buffer has been used by several investigators with good success.[59,136,137]

10.4.1.2.3 Trienzyme extractions. Folate extraction is often optimized from foods by the addition of α-amylase and/or protease digestion to the conjugase digestion protocol. Yamada,[138] in 1979, reported that total measurable folate could be significantly increased by addition of protease digestion during extraction to the traditional thermal extraction and conjugase digestion. The effect was demonstrated with human milk, hog liver, and cod muscle. Other researchers showed that addition of α-amylase digestion increased folate extraction from high starch and glycogen products.[139,140] Polysaccharide binding was demonstrated.[139] DeSouza and Eitenmiller[141] incorporated previous studies in development of a trienzyme approach to folate extraction applicable to most foods. The method substantially increased measurable folate from many food samples and was particularly effective on cereal-based and dairy products. The broad specificity protease from *Streptomyces griseus*, Pronase, and α-amylase together with chicken pancreas conjugase constituted the trienzyme treatment. The DeSouza and Eitenmiller procedure was adapted into analysis protocols at the Atlanta Center for Nutrient Analysis for folate analysis of Food and Drug Administration (FDA) Total Diet Market Basket Samples.[142]

Further application of the trienzyme method did not occur until Tamura et al.[136] used the trienzyme method with modified buffers and order of enzyme addition and showed significant increase in measurable folates in many food mixtures compared to traditional conjugase digestion. The suggestion was made that food composition databases should be revised using the trienzyme extraction. Such observations dramatically substantiate recommendations made by DeSouza and Eitenmiller[141] and Martin et al.[142] that traditional conjugase digestions may significantly underestimate the true folate value of the food supply. Another study has shown the applicability and worth of the trienzyme methodology for analysis of cereal-based foods. Pfeiffer et al.[137] modified the original methodology[141,142] by using HEPES/CHES buffer, pH 7.85, and rat plasma conjugase and quantitated the folate by LC. The trienzyme treatment led to variable increases of measured total folates in the products studied. Overall, significant increases as high as 34% were obtained. In addition, close agreement to microbiological results were obtained by the LC method when the trienzyme digests were assayed by both methods. The authors concluded that the trienzyme extraction is applicable to both LC and microbiological assays, and that traditional methods of extraction are not appropriate for folate assay of cereal-grain products. The Pfeiffer et al.[134] method is provided in Section 10.6.

With the need to accurately analyze total folate in the enriched cereal grain products, Rader et al.[17,18] documented the efficacy of the trienzyme extraction for analysis of total folate in fortified and nonfortified cereals. Modifications proposed by Rader et al.[17,18] were incorporated into a collaborative study on the trienzyme procedure completed by AOAC International[124] and the American Association of Cereal Chemists (AACC).[127,126] The method received First Approval Status by AACC and First Action Status by AOAC.[127] AOAC Official Method 2004.05, Total Folates in Cereals and Cereal Food, Microbiological Assay—Trienzyme Procedure (45.2.09)[122] is summarized in Section 10.7.

Chun et al.[143] provided a differential assay of folic acid and total folate in enriched cereals based on the *L. casei* ssp. *rhamnosus* microbiological assay. The folic acid was assayed by eliminating the protease and conjugase digestion steps of the trienzyme digestion. Since the

Table 10.12 Folic Acid, Total Folate, and Calculated Food Folate and μg Dietary Folate Equivalent (μg DFE) in Cereal-Grain Products and Processed Foods Containing Enriched Flour

	μg 100 g^{-1}			
Product	**Folic acid**	**Total folate**	**Food folate**	**μg DFE**
Wheat flour, white, unenriched (*n* = 24)	8[a]	31	23	37
Wheat flour, white, enriched (*n* = 46)	158	197	39	308
Bread, white (*n* = 23)	83	112	29	170
Rice, cooked (*n* = 13)	47	50	3	83
Pasta, cooked, various (*n* = 13)	64	76	12	121
Waffles/pancakes, cooked (*n* = 12)	54	66	12	104
Mixed dishes, cooked (*n* = 13)	17	24	7	36
Cakes, baked, various (*n* = 13)	17	21	4	33
Breakfast sandwiches, various (*n* = 30)	40	52	12	80
Toaster pastries, various (*n* = 8)	42	49	7	78
Macaroni and cheese, cooked (*n* = 13)	31	38	7	60
Macaroni, dry, enriched (*n* = 2)	243	274	31	444

[a] Mean.

Source: Chun, J., Martin, J. A., Chen, L., Lee, J., Ye, L., and Eitenmiller, R. R., A differential assay of folic acid and total folate in foods containing enriched cereal-grain products to calculate μg dietary folate equivalents (μg DFE), *J. Food Compos. Anal.*, 19, 182, 2006.

bacteria does not respond to folate containing more than three glutamic acid residues, the assay provided an accurate measure of folic acid. Use of the trienzyme procedure provided the total folate content and food folate was then calculated by difference. This procedure provides a fast and simple method to estimate μg DFEs in enriched cereal products. Data obtained by the differential assay for folic acid, total folate, food folate, and the μg DFE are shown in Table 10.12.

Since the initial publication of the trienzyme digestion by DeSouza and Eitenmiller,[141] various modifications of the procedure have appeared in the literature. pH effects, order of enzyme addition, incubation time, and other conditions that might effect the extraction of folate have been studied.[17,18,124,136,137,144,145,146–149] As originally reported by DeSouza and Eitenmiller,[141] trienzyme digestion does not increase measurable folate in all foods. Greatest effects were seen in dairy products and high starch containing products; however, protease digestion had minimal effects on extraction of folate from the high starch products. α-Amylase digestion is a necessary part of the extraction of folate from high starch products, since it decreases viscosity of the extracts, improves ease of handling, and generally, improves reproducibility of the assay.

With spinach, Pandrangi and LaBorde[147] found an optimum incubation time of 8 h for protease digestion and noted that α-amylase digestion did not appreciably affect measurable folate. Australian researchers were required for folate extraction from leafy vegetables and that trienzyme digestion actually decreased measurable folate. A similar observation was made by Shrestha et al.[150] and Iwatani et al.[151] who reported that α-amylase combined with conjugase gave higher results than the trienzyme digestion for breakfast cereals and Chinese broccoli. Such studies indicate that digestions can be specifically tailored to the food matrix to optimize folate extraction.

Chen and Eitenmiller[148] recently optimized the trienzyme digestion for vegetables using response surface methodology. Using CRM 485 mixed vegetables as the matrix, the optimal digestion times were 1.5 h for Pronase, 1.5 h for α-amylase, and 3 h for conjugase. Extended times past the optimal digestion times led to decreased measurable folate, which is understandable since longer incubation times for enzyme digestion increases the

exposure of folate to oxidation and other deleterious factors potentially present in the extraction media.

In other studies, Chen and Eitenmiller[149] completed a single laboratory method performance evaluation of the trienzyme extraction combined with a 96-well microplate assay. Using several standard reference materials as representative of a broad selection of food matrices, the results showed that the overall method provided a highly accurate, reproducible, and sensitive method for the assay of folate in a variety of foods. $HORRAT_r$ values of intra- and interassay precisions for all reference materials were within the acceptable range of 0.3–1.3.

Excellent reviews examining the trienzyme extraction have been prepared by Tamura[152] and Hyun and Tamura.[144] Several research papers based on microbiological assay of folate are summarized in Table 10.13.[132,140–143,153–173,145,151,146–149]

10.4.1.3 *Modification of traditional microbiological assays for folate*

Traditional microbiological assays have been modified to increase speed and decrease labor intensiveness. Microtiter 96-well plates and autoplate readers have been successfully used in several laboratories. In addition, rapid microbiological procedures based upon release of $^{14}CO_2$[157] and the use of CO_2[129] analyzers have been developed, which offer significant time savings. Such methods generally provide good agreement with traditional microassay techniques. Use of the microtiter plate assay should be seriously considered by all laboratories involved in microbiological vitamin analysis. Significant savings in labor and materials are obtainable. Detection limits are comparable to traditional methods as well as precision. Readers are encouraged to read Tamura's excellent review on use of the 96-well microplate assay.[174] Tamura's laboratory has been a pioneer in use of such assays. Techniques for the microplate assay are provided by Tamura.[174]

10.4.1.4 *Recommendations for the microbiological assay of folate*

1. The effects of trienzyme digestion should be established on foods considered for assay. Traditional extraction methods might not completely liberate folate from the food matrix. However, trienzyme digestion is not required for all foods; but, in many instances, significantly higher measurable folate can be obtained.[17,18,124,137,141,142,127,126,171,148,149] The trienzyme digestion can be optimized for specific matrices.[148]
2. The trienzyme extraction is the basis of AOAC Official Method 2004.05[122,124] and AACC Method 86-47.[127,126]
3. Extraction buffers based on the Wilson and Horne buffer, pH 7.85, or the phosphate buffer, pH 6.0, of Vahteristo et al.[59] are excellent stabilization systems. Both extractants contain ascorbic acid and 2-mercaptoethanol.
4. *L. casei* ssp. *rhamnosus* should be the assay organism for natural products. *E. hirae* is suitable for products containing only folic acid.
5. Microplate methods offer significant savings in time and materials.
6. When possible, microbiological assay results should be verified by LC or LC-MS procedures.
7. Traditional microbiological techniques can be modified to the microplate procedures.

Microbiological analysis of any water-soluble vitamin can be problematic. For folate in foods and biologicals, high variability among laboratories using microbiological analysis has been reported.[177] A study by Koontz et al.[177] compared total folate results from four commercial laboratories for analysis of nine foods including three certified reference materials. Between laboratory variation for folic acid-fortified foods (macaroni and pizza) was quite good (%CV—9–11). However, for nonfortified foods, between laboratory variation was

Table 10.13 Microbilogical Assay of Folate in Food and Biologicals

Matrix	Analyte	Buffer	Extraction		Detection	References
			Digestion	Microorganism		
1. Standards	$PteGlu_{1-7}$	0.05 M phosphate, pH 6.1, 0.1% AA	Hog kidney conjugase	*L. casei*	Turbidimetric	*Anal. Biochem.*, 49, 517, 1972[153]
2. Bread, flour	Total folate	0.1 M phosphate, pH 6.1 5 mg mL^{-1} AA	Hog kidney conjugase	*L. casei*	Turbidimetric	*Cereal Chem.*, 52, 348, 1975[154]
3. Plasma, erythrocytes	5-CH_3–H_4 folate	0.05 M phosphate, pH 6.1, 200 mg 100 mL^{-1} AA	—	*L. casei*	Radiometric	*J. Nucl. Med.*, 19, 906, 1978[155]
4. Spinach	Total folate	0.1 M phosphate, pH 7.0, 1% AA	Chicken pancreas conjugase	*L. casei*	Turbidimetric Radiometric	*J. Food Sci.*, 46, 552, 1981[156]
5. Various foods	Total folate	0.05 M phosphate, pH 6.1, 0.5 g 100 mL^{-1} AA	Chicken pancreas conjugase	*L. casei*	Radiometric	*J. Nutr.*, 113, 2192, 1983[157]
6. Various foods	Total folate	57 mM AA, pH 6.0	Hog kidney, chicken pancreas conjugase	*L. casei* Growth at pH 6.2 and 6.8	Turbidimetric	*Br. J. Nutr.*, 49, 181, 1983[158]
7–8. Spinach	Total folate	0.1 M phosphate, pH 7.0, 0.15% AA	Hog kidney, chicken pancreas conjugase	*L. casei*	Turbidimetric	*Nutr. Rep. Int.*, 28, 317, 1983;[159] *J. Food Sci.*, 49, 94, 1984[160]
9. Plasma	5-HCO–H_4 folate	0.1 M K_2HPO_4, pH 6.1, 0.1% AA	—	*L. casei* *P. acidilactici*	Turbidimetric Microtiter plate assay	*Anal. Biochem.*, 154, 509, 1986[130]
10. Various	Total folate	0.1 M NaOAC, pH 4.7, 5 g L^{-1} AA	Endogenous	*L. casei*	Turbidimetric	*J. Assoc. Off. Anal. Chem.*, 69, 773, 1986[161]
11. Spinach, broccoli	Total folate	0.1 M phosphate, pH 6.8, 1% AA	Chicken pancreas conjugase	*L. casei*	Turbidimetric	*J. Food Sci.*, 51, 626, 1986[162]
12. Food, plasma, diets, tissue	Total folate	Food—100 mM phosphate, pH 6.1, 0.5% AA Blood—50 mM phosphate, pH 6.1, 1% AA Orange juice—100 mM phosphate, pH 6.1, 0.2% AA	Hog kidney conjugase	*L. casei*	Turbidimetric	*J. Micronutr. Anal.*, 3, 55, 1987[163]

Continued

Table 10.13 (Continued)

Matrix	Analyte	Buffer	Extraction			Reference
			Digestion	Microorganism	Detection	
13. Various foods	Total folate	0.2 M phosphate, pH 6.1, 5 g AA	Chicken pancreas, hog kidney conjugase	*L. rhamnosus*	Turbidimetric	*Br. J. Nutr.*, 59, 261, 1988[140]
14. Standards	PteGlu,H_4 folate H_2 folate 5-CH_3-H_4 folate 5-CHO-H_2 folate 10-CHO-H_2 folate	pH 6.1, 0.05 M phosphate buffer, 0.15% AA		*L. casei*	CO_2 analyzer Turbidimetric	*J. Micronutr. Anal.*, 6, 19, 1989[129]
15–20. Infant formula, baby foods, cowpea flour, various foods, feeds	Total folate	—0.1 M sodium phosphate (dibasic), 1% AA, pH 6.8 16.—1.42% sodium phosphate, 1% AA, pH 6.8	Chicken pancreas Pronase®, α-amylase trienzyme	*L. casei*	Turbidimetric	*J. Micronutr. Anal.*, 7, 37, 1990;[142] *J. Assoc. Off. Anal. Chem.*, 73, 805, 1990;[143] *J. Sci. Food Agric.*, 64, 389, 1994;[164] *Poultry Sci.*, 74, 1447, 1995;[145] *Poultry Sci.*, 74, 1456, 1995;[166] *J. Food Sci.*, 61, 1039, 1996[167]
21. Standards	$PteGlu_{1-7}$	—0.1 M phosphate buffer, 0.17% AA, pH 6.0, 6.5, 7.2 for plasma conjugase —0.1 M phosphate buffer, 0.1% AA, pH 7.2 for chicken pancreas	Human blood plasma, chicken pancreas conjugase	*L. casei*	CO_2 analyzer	*Food Chem.*, 43, 57, 1992[132]
22. Serum and red cells	Total folate	0.5% sodium ascorbate		*L. casei*	Microtiter plate technique Turbidimetric	*J. Clin. Pathol.*, 45, 344, 1992[168]
23. Infant formula	Total folate	0.05 M phosphate buffer 0.05 M phosphate buffer + 0.05% AA	Chicken pancreas conjugase	*L. casei*	Titrimetric Turbidimetric	*J. AOAC Int.*, 76, 399, 1993[123]
24. Multi-vitamin formulations	PteGlu	A—0.1 M phosphate buffer, pH 8.0 B—0.05 M phosphate buffer, 0.05% AA, pH 6.1		*S. faecalis*	Microplate	*J. AOAC Int.*, 78, 1173, 1995[169]

25. Freeze dried space shuttle foods, frozen vegetables	Total folate	50 mM Hepes/Ches buffer, pH 7.85	Chicken pancreas conjugase	*L. casei*	Microplate	*J. Food Sci.*, 60, 538, 1995[170]
26. Mixed foods	Total folate	A0.1 M potassium phosphate buffer, 57 mM AA, pH 6.3	Chicken pancreas conjugase	*L. casei*	Microplate Turbidimetric	*J. Agric. Food Chem.*, 45, 135, 1997[136]
27. Cereal-grain products	Total folate 5-CH_3–H_2 folate 10-CHO–H_2 folate 10-CHO–folate 5-CHO–H_2 folate PteGlu	50 mM Hepes/Ches buffer, pH 7.85 containing 2% (W/V) AA and 10 mM 2-MCE	Rat plasma conjugase	*L. rhamnosus*	PDA, HPLC Turbidimetric	*J. Agric. Food Chem.*, 45, 407, 1997[137]
28. Human milk, dairy products, various foods	Total folate	Various buffers	Trienzyme	*L. casei*	Microplate Turbidimetric	*Food Chem.*, 63, 401, 1998;[171] J. Nutr. Sci. Vitaminol., 44, 361, 1998;[148] J. Food Sci., 67, 817, 2001;[172] Food Res. Intl., 35, 565, 2002[173]
29. Enriched cereal-grains	Total folate	Phosphate buffers, various pH levels (pH 4.3–7.8)	Traditional and Trienzyme	*L. casei*	Turbidimetric	Food Chem., 62, 451, 1998;[17] Food Chem., 70, 275, 2000[18]
30. Various foods	Total folate	Phosphate-ascorbate, pH 7.2 for chicken pancreas, phosphate-ascorbate, pH 4.5 with 100 mM mercapto-ethanol for human plasma conjugase	Traditional and Trienzyme	*L. casei*	Turbidimetric	*Food Chem.*, 71, 545, 2000;[150] *J. Food Compos. Anal.*, 16, 37, 2003[151]
31. Enriched cereals	Total folate	Phosphate-ascorbate, pH 7.2	Traditional and Trienzyme	*L. casei*	Turbidimetric	*Cereal Food World*, 46, 216, 2001;[127] *AAAC*, 10th ed., 2000[126] (Method 86-47)
32. Pulses	Total folate	AAAC 86-47	Trienzyme	*L. casei*	Turbidimetric	*J. Agric. Food Chem.*, 51, 5315, 2002[146]
33. Spinach	Total folate	Various buffers and pH ranges	Trienzyme	*L. casei*	Turbidimetric Microplate	*Intl. J. Food Sci. Technol.*, 39, 525, 2004[147]
34. Cereal grain products	Folic acid Total folate	Phosphate-ascorbate, pH 7.8	Trienzyme	*L. casei*	Turbidimetric Microplate	*J. Food Compos. Anal.*, 19, 182, 2006[143]

high (%CV > 45). Further, high within laboratory variation was noted (%CV—11–27). Variability was attributed to variation in extraction methods used by the laboratories, failure to employ the trienzyme digestion, differences in sample storage conditions, possibility of incomplete deconjugation of polyglutamates, and differences in growth conditions for microbiological assay between laboratories. The authors also noted that equal response of *L. casei* ssp. *rhamnosus* to folic acid and 5-methyltetrahydrofolate, the most commonly present folate form in foods, is not guaranteed. In this regard, researchers following the AOAC International protocol that specifies folic acid as the standard should document the response of *L. casei* ssp. *rhamnosus* to 5-methyltetrahydrofolate under the specific assay conditions employed in the laboratory. The study convincingly showed the difficulties encountered in microbiological analysis of folate in biological matrices and lack of application of standard assay protocols.

A recent review of methods of analysis for folate reemphasized the fact that microbiological assay of folate is still the most commonly used method to determine folate content of foods.[178] While LC-MS procedures offer many advantages, instrument cost and required expertise of the analyst puts the methodology out of reach of many laboratories worldwide. Although problematic, microbiological assay by validated methods such as AOAC Official Method 2004.05 will continue to provide significant information on the folate content of the world's food supply. Studies designed to standardize microbiological methods for folate analysis are, therefore, still needed to improve overall quality of the data obtained by microbiological assay.

10.4.1.5 AOAC International Official Methods

AOAC International[122] does not provide LC methods for folate assay. Methods currently available use either *E. hirae* (*S. faecium*) ATCC No. 8043 or *L. casei* ssp. *rhamnosus* ATCC No. 7469 for microbiological assay.

AOAC Official Method 944.12, Folic Acid (Pteroylglutamic Acid) in Vitamin Preparations, Microbiological Method, AOAC Official Methods of Analysis, 45.2.03.

Method 944.12 uses the response of *E. hirae* for measurement of folic acid in pharmaceutical products formulated with folic acid. The method is not designed for assay of naturally occurring forms because *E. hirae* does not respond to 5-CH_3-H_4 folates or other coenzyme forms. In addition, the method does not provide extraction conditions necessary for extraction and deconjugation of poly-γ-glutamyl folates. The extraction procedure for vitamin preparations has the following steps:

1. Add water equal in mL to greater than or equal to ten times the dry weight of sample. Folic acid concentration must be less than or equal to 1.0 mg folic acid per mL.
2. Add equivalent of 2 mL NH_4OH (2 + 3) per 100 mL liquid.
3. Disperse, wash down sides of flask with 0.1 N NH_4OH.
4. Autoclave, 121°C–123°C, 15 min.
5. Cool and filter or centrifuge.
6. Dilute, adjust to pH 6.8.

AOAC Official Method 992.05, Folic Acid (Pteroylglutamic Acid) in Infant Formula, Microbiological Method, AOAC Official Methods of Analysis, 50.1.21.

Method 992.05 is applicable only to the assay of folic acid in infant formula. *L. casei* ssp. *rhamnosus* is the assay organism. While the method is not intended for measurement of total folate, a conjugase digestion step is included in the extraction, so the method undoubtedly measures folate originating from natural ingredients.[124] Extraction steps include the following:

1. Reconstitute or dilute formula with water to a concentration of ca 5 μg folic acid per 100 mL.

2. Pipet 1 mL diluted sample and 1 mL chicken pancreas preparation into 18 mm × 150 mm screw top culture tubes, mix. Add 18 mL 0.05 M phosphate–ascorbate buffer (50 mg ascorbic acid 100 mL^{-1}).
3. Add 1 mL toluene.
4. Incubate for 16 h at 37°C.
5. Autoclave, 121°C, 10 min.
6. Centrifuge.
7. Dilute with 0.05 M phosphate–ascorbate buffer to required folic acid concentration.

AOAC Official Method 2004.05, Total Folates in Cereals and Cereal Foods, Microbiological Assay, Trienzyme Method, accepted First Action 2004 measures total folate including folic acid in cereals and enriched cereal grain products.[124] The method is similar to AACC Method 86-47.[127,126] Although applicable only to cereal products, the method incorporates the trienzyme digestion, which is universally useful for total folate assay in all foods and has been used as such for the past decade. The trienzyme digestion as presented in the AOAC Official Method 2004.05 is summarized in Section 10.6.

The trienzyme extraction increases the amount of measurable folate in many foods compared with the traditional conjugase digestion; however, as previously discussed in Section 10.5.1.2.3, the effects cannot be predicted by food matrix type. In addition, α-amylase and/or protease digestion is not required for all matrices. Generally, application of the trienzyme digestion is most effective on dairy foods and cereal products as first recommended by DeSouza and Eitenmiller.[141]

10.4.2 Ligand-binding assays

Various biospecific methods using ligand binding have been developed for folate analysis of clinical and food samples. Biospecific methods for water-soluble vitamins were classified into two groups by Finglas and Morgan.[179] Methods based on the specific interaction of antibody with its antigen include RIA and enzyme-linked immunoabsorbent assay (ELISA). Assays using naturally occurring vitamin binding proteins with isotope labels or enzyme labels include radiolabeled protein binding assays (RPBA) based on isotope dilution procedures and enzyme protein binding assays (EPBA).

Folate assay methods have been developed using each of these ligand binding approaches. RPBA procedures and, to a limited extent, EPBA methods are used for folate assay of clinical samples. Such methods have largely replaced use of microbiological methods for the assay of folate in blood and serum. Because of the significance to clinical laboratory operations, the performance of the most widely used techniques have been extensively evaluated and compared to microbiological and LC methods for the analysis of serum, blood, and erythrocyte folate levels. Two such extensive studies of commercial RPBA kits were conducted by the Center for Food Safety and Applied Nutrition (CFSAN), FDA, and Centers for Disease Control (CDC) in the United States, and by the Food-Linked Agro-Industrial Research Program (FLAIR) in the European Community. These studies have been widely discussed in the scientific literature. Reports that analysts should read before attempting to initiate blood analysis using RPBA techniques include conclusive papers on both the United States and European Community studies.[179,180,181] Both reports discussed problems with standards used for calibration of available kits up to 1994. Overestimation of serum and red blood cell folate values occurred over several years and produced questionable data regarding folate status. Since the commercial kits were used in the U.S. National Health and Nutrition Surveys (II and III), the calibration problems led to the extensive study. FLAIR comparative studies showed that commercial diagnostic kits were subject to between-kit and between-laboratory variability. Also, RPBA results often did not compare with LC or microbiological analyses. FLAIR recommendations include the

need for further standardization and optimization of assay and extraction procedures, the need for appropriate, clinically and nutritionally relevant reference materials, and more extensive information on kit calibrants from manufacturers. Further, laboratories using the kits should verify reference standards, before use, by use of pure, calibrated standards, and in-house quality control samples. All can learn from the European and U.S. experiences earlier in this decade. Quality assurance programs are now demanding proper control of problems associated with the extensive use of RPBA kits in clinical situations.

Wigertz and Jägerstad[182] compared LC folate analysis of milk and blood to data obtained by a commercial RPBA assay. Their data showed that the RPBA method overestimated H_4 folate. Plasma folate values were higher as measured by RPBA and compared to LC values. LC profiles showed only 5-methyltetrahydrofolate in the plasma. Overestimation by RPBA was thought to be owing to the use of folic acid rather than 5-methyltetrahydrofolate as the calibrating standard. It has been known for many years that milk folate-binding protein has greater affinity for folic acid than for 5-methyl-tetrahydrofolate between pH 7.2 and 8.0. At pH 9.3, binding affinity was equal.[48]

Use of competitive binding assays for food analysis have been hampered by problems arising from variation of ligand binding affinity for the multiple folate forms present in many foods.[48,117] Gregory[48] stated the following, "On the basis of the known specificity of the bovine milk folate-binding protein, it is difficult to rationalize studies reporting good correlation between the results of competitive binding and microbiological assays." Even if the RPBA is completed at pH 9.3, RPBA results frequently do not compare to microbiological assay results. DeSouza and Eitenmiller[141] found good agreement between RPBA and microbiological assay of infant formula, which contains predominantly folic acid added during manufacture, but poor agreement for assay of baby foods containing natural folates. Such differences in results by microbiological and radioassay procedures are common throughout the literature on application of RPBA to food analysis. Comparison of LC, microbiological, and RPBA assays of milk show close agreement.[181,182,183] This is expected since up to 95% of the folate in milk is 5-methyltetrahydrofolate.

Additional RPBA studies have shown that RPBA is suitable for food analysis if 5-CH_3H_4 folate is the primary form in the commodity.[183,184] However, matrix effects can lead to significant false response from components in the food other than folate. Strälsjo et al.[184] showed that, for various berries, false RPBA response could be greater than 50% owing to nonspecific binding. For high folate-containing products such as strawberry, rose hips, and blackberries, the false response was relatively minor (<7%); however, for low folate products such as black currant and blueberries, the false response approached 50% or greater. Strälsjo's research[183,184] reinforced general lack of confidence for ligand binding assays for food analysis, unless the data is confirmed by other analytical methods. RPBA methods should be considered only complimentary to other assay methodologies for folate analysis of food.

Another approach to FBPA for food analysis has been developed by Finglas et al.[185,186] The method utilized a folate-binding protein-peroxidase conjugate and did not use isotopes. The EPBA assay showed similar responses for 5-formyltetrahydrofolate and 5-methyltetrahydrofolate. The authors stressed that folic acid must be used for calibration if fortified foods are assayed. The method was used to assay raw and cooked vegetables with good comparative results to microbiological assay by *L. rhamnosus*. Analysis time was four hours by EPBA compared to 2–3 days for the microbiological assay. The method was included in a Community Bureau of Reference of the Commission of the European Communities (BRC) comparative study.[187] Results between laboratories varied from 378 to 1051 mg 100 g^{-1} when human plasma conjugase was used in the analysis with EPBA assay of a reference brussels sprout sample. Similar large variations occurred with use of chicken pancreas conjugase. The conclusion was that EPBA and RPBA methods for food analysis are

limited by the response of the binding protein to natural folate forms, and that application of the ligand binding methods "may yield tenuous results." Failure to obtain acceptable ligand binding assays for folate analysis of foods leads to the need for good LC methods. As discussed in the next section, LC methods have been significantly improved and can be routinely applied in food analysis programs for folate.

10.4.3 Advances in the analysis of folate and folic acid

10.4.3.1 High performance liquid chromatography and high performance liquid chromatography-mass spectrometry

Liquid chromatography (LC) methods for folate analysis have been refined to the point that most analytical laboratories equipped with modern LC systems and detectors, specifically a fluorescent or photodiode array detector, can successfully assay naturally occurring folates. The primary advantage of LC analysis is the ability to quantify the specific folate forms and, if so desired, the γ-glutamylfolate polymers. Such specificity is not obtainable by other methods.

Over the last decade, LC combined with mass spectrometry (LC-MS) has added a new dimension to folic acid-folate analysis. The instrumentation developed for LC-MS has rapidly improved to the point that these methods are undoubtedly the method-of-choice for folate assay. Specificity and sensitivity provided by MS detection takes estimation and lack of specificity out of the folate analysis picture. It not only provides accurate data for total folate, but also accurate data for the coenzyme constituents. Unfortunately, cost associated with LC-MS instruments puts the methodology out of reach of many investigators. Indeed, microbiological assays for total folate acid will be a significant source of folate data for years to come. Liquid chromatographic methods, even without MS detection, are far superior to microbiological methods, but remain out of reach of some laboratories.

Our attention will be given to a few recently published methods that have advanced the LC analysis of folate. In addition, method summaries provided in Table 10.14 give details of extraction methods and LC chromatography and detection parameters. The papers included in Table 10.14 were chosen from the standpoint to provide an historical perspective of method development for the LC analysis of folate in food and clinical samples and to provide information on LC-MS methodology. The methods were carefully chosen from quite a large pool of LC method development papers on folate analysis.[133,58,59,137,188–257]

10.4.3.1.1 Folate extraction for LC analysis. Procedures for extraction of folate have their basis in studies completed for microbiological assay in earlier decades and more recently with the acceptance of the trienzyme extraction. Such information discussed in Section 10.5.1.2 has been refined for preparatory methods useful for LC analysis. Procedural steps include the following:

1. *Dispersion or Homogenization in a Stabilizing Buffer*: Buffer systems were discussed in Section 10.5.1.2 for microbiological assays. Buffers must contain a stabilizing agent to protect labile folates from oxidative degradation. For food analysis, the addition of ascorbic acid and 2-mercaptoethanol has been recommended.[59,137] The Wilson and Horne buffer contains 2% ascorbate and 0.2 M 2-mercaptoethanol. Other researchers use only ascorbic acid with good results.[179,199] Ascorbic acid cannot be used with EC detection owing to high background current.[236] Lucock et al.[58,236] recommended dithiothreitol for stabilization of plasma in conjunction with EC detection. Lucock et al.[241] indicated that ascorbic acid and dithiothreitol are suitable antioxidants for most biological samples depending on detection systems and pH conditions. Thus, these antioxidants should be used selectively. Ascorbic acid is

Table 10.14 LC and LC-MS Methods for the Analysis of Folates in Foods, Pharmaceuticals, and Biologicals

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods					
Fortified cereal, beef liver/Folic acid, 5-CH_3-H_4 folate, H_2-folate, 5-CHO-H_4 folate, 10-CHO-H_4 folate	Infant formula—dilute w/0.1 M KH_2PO_4, 0.25% AA, pH 7.0, adjust pH to 4.5, centrifuge, filter. Cereal—Homogenize in 0.1 M KH_2PO_4, 0.25% AA, pH 7.0, centrifuge, filter. Liver—Homogenize in 0.1 M KOAC, 0.25% AA, pH 4.0, incubate, 37°C, 2 h. Heat, 100°C, 10 min, centrifuge Clean up—liver: Bio-Beads SM-2	Ultrasphere IP, 25 cm × 4.6 mm in eseries w/μBondpak phenyl 30 cm × 3.9 mm Precolumn, silica 25 cm × 4.6 mm Mobile phase—isocratic 0.033 M KH_2PO_4:MeCN (91.5:9.5), pH 2.3 Flow rate—0.7 mL min^{-1}	Post-column derivatization Fluorescence Ex λ = 365 Em λ = 415 or UV 280 nm	DL (ng) on-column)— 2–40 (UV) 0.2–0.9 (fluorescence) %Recovery—65–82	*J. Agric. Food Chem.*, 29, 374, 1981;[188] *J. Food Sci.*, 47, 1568, 1982[189]
Pharmaceutical, infant formula, meal replacer, egg replacer/ folic acid	Pharmaceutical—extract w/0.05 M phosphate/citrate buffer, pH 8.0, containing 0.5 mg mL^{-1} AA. Infant formula—disperse in water, dilute w/phosphate/citrate/ AA buffer. Digest w/papain, 40°C, <4 h or overnight, centrifuge and filter Clean up—Infant formula: DEAE cellulose	Spherisorb ODS, 10 μm, 25 cm × 4.6 mm Mobile phase—gradient A—0.1 M NaOAC (pH 4.0): MeCN (98:2) B—0.1 M NaOAC (pH 4.0): MeCN (70:30) Flow rate—1 mL min^{-1}	280 nm	DL—(on-column)—4.6 ng %CV—5.6–6.8 %Recovery—96–98	*J. Liq. Chromatogr.*, 5, 953, 1982[190]
Commercial diets/ folic acid	Disperse in 0.01 M phosphate, pH 7.4, filter. Clean up—SPE: Bond Elute, Quaternary amino anion-exchange	μBondapak C Mobile phase—isocratic 0.01 M NaOAC, pH 5.7: MeCN (94:6) Flow rate—2 mL min^{-1}	365 nm or PDA	%Recovery—100–102	*J. Liq. Chromatogr.*, 7, 2659, 1984[191]

Milk, dairy products/ folic acid, H_2 folate, H_4 folate, 5-CHO-H_4 folate, 10-CHO-H_4 folate	Adjust pH to 4.5 w/HAC. Homogenize, centrifuge. Decant supernatant. Add phosphate buffer, pH 4.5 containing 10% AA and 1 M 2-MCE to concentration of 0.1% AA and 0.1 M 2-MCE. Digest w/HK conjugase, 37°C, 2 h. Centrifuge and filter	C_{18} Microsorb, 3 µm, 10 cm × 4.6 mm Mobile phase—isocratic Phosphate buffer, pH 6.8: MeOH (50:50) containing 50 mL L^{-1} 1.0 M TBAP Flow rate—1 mL min^{-1}	EC +900 mV versus Ag/AgCl or Fluorescence Ex λ = 238 Em λ = 340	QL— 0.3–7.3 ng g^{-1}	*J. Chromatogr.*, 449, 271, 1988[192]
Various foods/ polyglutamyl folates 1-7, folic acid 1-7, H_2 folate 1-7, H_4 folate 1-7, 5-CH_3-H_4 folate 1-7, 5-CHO-H_4 folate 1-7, 10-CHO-H_4 folate 1-7	Suspend or dilute in solution of 2% AA. 10 mM 2-MCE and 100 mM Bis-tris, pH 7.8 in autoclave, 30 min. Cool, homogenize, centrifuge. Mix supernatant w/[^{3}H] folic acid. Clean up—folate binding protein-Sepharose 4B affinity column	Econosphere, 5 µm, 10 cm × 4.6 mm Mobile phase—gradient A—5 mM TBAP, 0.5 mM DET in 25 mM phosphate/Tris buffer, pH 7.4 or 25 mM NaCl in water B—5 mM TBAP, 0.5 mM DET in 25 mM phosphate/Tris buffer, pH 7.4 in MeCN: EtOH:water (64:9:27) Flow rate—1 mL min^{-1}	PDA 10-CHO-H_4— 350 nm folate—258 nm	%CV—5–19	*Anal. Biochem.*, 182, 94, 1989;[193] *J. Nutr. Biochem.*, 4, 488, 1993[194]
Various foods/ Folic acid, $PreGlu_{1-3}$	Homogenize in 50 mM NaOAC, pH 4.9, containing 50.5 mM AA. Flash w/N_2, seal. Boil, 60 min, cool. Centrifuge, adjust pH to 4.9, filter. Digest w/HK conjugase	Perkin Elmer, 3 × 3 C_{18} Mobile phase—isocratic 0.1 M KOAC, pH 5.0, containing 1.2% MeCN Flow rate—1.5 mL min^{-1}	Post-column derivatization Fluorescence Ex λ = 365 Em λ = 415	—	*J. Agric. Food Chem.*, 38, 154, 1990[195]
Citrus juice/ 5-CH_3-H_4 folate	Centrifuge diluted juice, adjust pH to 5.0. Digest w/HK conjugase, 37°C, 90 min. Cool, centrifuge, filter. Clean up—SPE Bond Elut, Phenyl pre-column back flushed to analytical column	Zorbax ODS C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—Gradient A—MeOH:phosphate/acetate buffer, pH 5.0 (10:90) containing 0.005 M TBAP B—MeOH:phosphate/acetate buffer, pH 5.0 (30:70) containing 0.005 M TBAP Flow rate—1 mL min^{-1}	EC +200 mV vs Ag/AgCl	%CV—2.8	*J. Agric. Food Chem.*, 38, 1515, 1990[196] & 39, 714, 1991[197]

Continued

Table 10.14 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods (Continued)					
Elemental diets/ Folic acid	Dissolve in water containing NaCl (10 g 60 mL^{-1}), 50°C. Allow to stand 30 min at ambient temperature. Dilute to 100 mL w/water. Extract w/Hex, inject aqueous layer	Capcellpak C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:water (9:91) containing 1 mM Na_2EDTA Flow rate—1 mL min^{-1}	360 nm	%Recovery—95	*J. Chromatogr.*, 609, 399, 1992[198]
Milk, whole blood, plasma, various foods/folic acid, H_4 folate, 5-CH_3-H_4 folate, 5-CHO-H_4 folate, 10-CHO folic acid	Extract samples w/5-6 vol 75 mM KH_2PO_4 containing 52 mM AA and 0.1% 2-MCE, pH 6.0, homogenize under N_2. Add 2-octanol to reduce foaming. Microwave 1 min, boiling water bath, 10 min, cool. Centrifuge, redissolve residue w/extraction buffer, centrifuge, combine extract, adjust aliquot to pH 4.9. Digest w/HK conjugase, 37°C, 2 h. Boiling water bath, 5 min. Clean up—SPE, Baker quaternary amine (N^+) strong anion-exchange	Hypersil ODS, 3 µm, 15 cm × 4.6 mm Mobile phase—gradient MeCN:30 mM KH_2PO_4, pH 2.0 Flow rate—0.8 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 356 10-CHO-H_4 folate Ex λ = 360 Em λ = 460	DL (on-column)—0.03–0.1 ng %CV—2.4–11.6 %Recovery—50–90	*Food Chem.*, 57, 109, 1996[199] & 59, 589, 1997;[200] *J. Agric. Food Chem.*, 44, 477, 1996;[59] *J. Food Sci.*, 61, 524, 199a6;[201] *J. AOAC Int.*, 80, 373, 1997;[202] *J. Chromatogr.*, 855, 237, 1999;[203] *J. Sci. Food Agric.*, 81, 938, 2001[204]
Cereal foods/ Folic acid, 10-CHO-folate, 5-CHO-H_4 folate, 10-CHO-H_2 folate, 10-CHO-folic acid, 5-CH_3-H_4 folate	Add 10 vol HEPES/CHES buffer, pH 7.85 (50 mM HEPES, 50 mM CHES) containing 2% AA and 10 mM 2-MCE, vortex, boiling water bath, 10 min. Cool, homogenize. Digest rat plasma conjugase and α-amylase (37°C, 4 h) followed by protease (37°C, 1 h). Boiling water bath, 5 min, cool. Centrifuge, resuspend residue in extraction buffer, centrifuge, filter Clean up—Folate binding protein-Affigel	Ultremex C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—gradient A—MeCN B—0.033 M phosphoric acid, pH 2.3 Flow rate—1 mL min^{-1}	PDA UV 280 nm	%Recovery—87–107	*J. Agric Food Chem.*, 45, 407, 1997[137]

Fortified fruit juice/ Folic acid	Clean up—Chromabond-SB cartridges w/strong anion exchange material	Eurospher 100 C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient A—5 mM TBAHS—25 mM NaCl in water B—5 mM TBAHS-25 mM NaCl-1 mM KH_2PO_4: MeCN (35:65) Flow rate—1 mL min^{-1}	PDA UV 284 nm	DL—0.04 mg L^{-1} QL—0.06 mg L^{-1} %Recovery—78–93 %RSD <2.3	*Food Chem.*, 74, 521, 2001[205]
Various foods/H_4 folate, 5-CH_3-H_4 folate, 10-HCO H_2 folate, 10-CHO-Folic acid, 5-CHO-H_4 folate, folic acid	Add 40 mL 0.05 M HEPES/CHES buffer, pH 7.8 (containing 2% AA and 0.01 M 2-MCE) to sample, vortex, boiling water bath, 10 min. Cool. Homogenize. Apply trienzyme treatment to the sample suspensions. Clean up—affinity chromatography	Vydac 201TP 54 C_{18}, 5 μm, 25 × 4.6 mm Mobile phase—gradient Phosphate buffer (0.033 M, pH 2.1) - MeCN Flow rate—0.8 mL min^{-1}	PDA 280 nm Fluorescence Ex λ = 280 Em λ = 359 and Ex λ = 360 Em λ = 460	%RSD—3.3–21.0 %Recovery—73–109	*J. AOAC Int.*, 82, 119, 1999;[206] *Am J. Clin. Nutr.*, 73, 765, 2001[207]
Rye/H_4 folate, 5-CH_3-H_4 folate, 10-HCO H_2 folate, 5-CHO-H_4 folate, Folic acid, 10-HCO-folate	Add 35 mL 0.05 M HEPES/CHES buffer, pH 7.8 (containing 2% AA and 0.01 M 2-MCE) to 1-2 g milled sample, flush w/N_2, boiling water bath, 10 min. Cool. Apply trienzyme treatment to the sample suspensions. Clean up—affinity chromatography	Hypersil ODS, 3 μm, 15 cm × 4.6 mm Mobile phase—gradient MeCN—30 mM phosphate buffer, pH 2.2 Flow rate—0.9 mL $^{-1}$	UV 290 nm Fluorescence 10-HCO-folate Ex λ = 360 Em λ = 460 others Ex λ = 290 Em λ = 356	DL—0.04–0.9 ng $injection^{-1}$ %CV—8	*J. Sci. Food Agric.*, 81, 938, 2001[208]
Vegetables/H_4 folate, 5-CH_3-H_4 folate, 5-CHO-H_4 folate, 10-CHO-H_4 folate, folic acid, 5,10-CH_2 H_4 folate, H_2-folate	Extract samples 30 mL 100 mM phosphate buffer containing 1.0% AA (pH 6.0), homogenize under N_2. Add 2-octanol to reduce foaming. Boiling water bath, 100°C, 10 min, cool. Filter under vaccum. Redissolve residue w/extraction buffer, combine extract, dilute to 50 mL w/extraction buffer. Clean up—Affinity chromatography, FBP column	Purospher Star RP-18, 5 μm, 15 cm × 4.6 mm Mobile phase—gradient A—0.01 M acetate buffer (pH 4.75):MeCN (98:2) B—0.01 M acetate buffer (pH 4.75):MeCN (68:32) Flow rate—0.7 mL min^{-1}	Post-column derivatization Fluorescence Ex λ = 420 Em λ = 485	$\%RSD_{intra}$—4.9–11.4 $\%RSD_{inter}$—5.8	*J. Agric. Food Chem.*, 51, 7872, 2003[209]

Continued

Table 10.14 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods (Continued)					
Raw and processed beetroots, peas/ H_4 folate, 5-CH_3-H_4 folate, 5-CHO-H_4 folate	Extract samples w/20 mL 0.1 M phosphate buffer containing 2% AA and 0.1% 2-MCE, pH 6.0, homogenize under N_2. Add 2-octanol to reduce foaming. Boiling water bath, 10 min, cool. Adjust pH to 4.9. Digest w/HK conjugase, 37°C, 3 h. Boiling water bath, 5 min. Cool, centrifuge. Centrifuge, redissolve residue w/extraction buffer, centrifuge, combine extract Clean up—SPE (SAX) cartridge	LiChrospher 100 RP-18, 5 μm, 12.5 cm × 4.0 mm and Zorbax SB C_8, 5 μm, 15 cm × 4.6 mm Mobile phase—gradient A: 30 mM KH_2PO_4 (pH 2.3) B: MeCN Flow rate—0.4 mL min^{-1}	UV 290 nm Fluorescence Ex λ = 290 Em λ = 360	DL—0.07–1.5 ng mL^{-1} QL—0.2–4.0 ng mL^{-1} $\%RSD_{intra}$—0.9–5.6 $\%RSD_{inter}$—1.0–8.1 %Recovery—85–97	*Food Chem.*, 101, 1095, 2006[64] & 80, 579, 2003[210]
Tempe/5-CH_3-H_4 folate, 5-CHO-H_4 folate, 10-CHO-H_4 folate, folic acid	Add 40 mL 0.05 M HEPES/CHES buffer, pH 7.85 (containing 2% AA and 0.01 M 2-MCE, vortex, boiling water bath, 10 min. Cool. Apply trienzyme treatment to the sample suspensions. Clean up—SPE (SAX) cartridge	Phenomenex C_{18}, 5 μm, 12 cm × 4.6 mm Mobile phase—gradient A—30 mM KH_2PO_4 (pH 2.2) B—MeCN Flow rate—0.8 mL min^{-1}	UV 290 nm	%Recovery—80–109	*J. Agric. Food Chem.*, 52, 7752, 2004[211]
Cereals, cereal-grain products/5-CH_3-H_4 folate, 10-HCO H_2 folate, 10-HCO-folate, 5-HCO-H_4 folate, Folic acid	Add 20 mL HEPES/CHES buffer, pH 7.85 containing 2% AA and 0.2 M 2-MCE to 2 g sample, homogenize, flush w/N_2, boiling water bath, 10 min. Cool. Apply trienzyme treatment to the homogenate Clean up—affinity chromatography	Phenomenex Luna C_{18} 5 μm, 25 cm × 4.6 mm Mobile phase—gradient MeCN—33 mM phosphate buffer, pH 2.3 Flow rate—1 mL min^{-1}	UV 290 nm Fluorescence Ex λ = 290 Em λ = 356	%Recovery—78–104	*Eur. Food Res.* Technol., 22, 208, 2005[212]

Fresh frozen fruits and vegetables/ 5-CH_3-H_4 folate	Homogenize in 1 M KH_2PO_4, 10 mM AA, 10 mM 2-MCE buffer, pH 6. Modified trienzyme digestion followed by clean up on strong anion exchange SPE (extract clean SAX)	HSC 18, 3 μm, 15 cm × 4.6 mm Mobile phase—gradient Phosphate buffer (33 mM, pH 2.2):MeCN 0-30% MeCN Flow rate—0.8 mL min^{-1}	PDA 280 nm and 350 nm Fluorescence Ex λ = 290 Em λ = 350	Data verified by LC-MS	*Food Chem.*, 92, 587, 2005[77]
LC-MS					
Foods/H_4 folate, 5-CH_3-H_4 folate, 5-CHO-H_4 folate, folic acid	Add 10 mL 0.1 M NH_4OAC containing 1% AA, pH 4, 0.1% 2-MCE to 0.01—1 g sample. Boiling water, 1 h, shake every 15 min. Cool, centrifuge. Clean up—SPE C_{18}	Hypersil ODS 3 μm, 12.5 cm × 3 mm Mobile phase—isocratic 2.5 mM HAC:MeCN (88:12) Flow rate—0.5 mL min^{-1}	MS—ESI negative ion mode w/SIM	—	*J. Chromatogr. A*, 864, 59, 1999[213]
Fortified foods/ folic acid	Homogenize 5g sample w/100 mL 0.03 M K_2HPO_4, 0.1% 2-MCE, pH 3.5. ^{13}C-labeled folic acid as an internal standard. Purge w/N_2, boiling water bath, 100°C, 1 h, cool. Add 1.5 mL EtOH to 10 mL supernatant, vortex, 1 min. Stand overnight, centrifuge. Clean up—SPE	Phenomenex C_{18} 5μm 15 cm × 4.5 mm Mobile phase—isocratic Water:MeCN-water-MeOH (26-60-14), 0.1% formic acid (40:60) Flow rate—0.2 mL min^{-1}	MS—ESI negative ion mode w/SIM	%RSD—3.8	*J. Agric. Food Chem.*, 49, 1282, 2001;[214] *J. Food Compos. Anal.*, 16, 281, 2003[215]
Citrus juices/ Folic acid, 5-CH_3-H_4 folate	Mix 4 mL sample w/20 mL 0.1 M K_2HPO_4 containing 10 mM MCE, AA and 10 mg L^{-1} NaN_3. Adjust pH to 6.0. Add 2-octanol and 100 μL rat plasma, homogenize. Incubate, 37°C, 16 h. Centrifuge, adjust pH to 3.0. ^{13}C-labeled folic acid as an internal standard. Clean up—SPE	Phenomenex C_{18}, 5 μm, 15 cm × 4.6 mm Mobile phase—gradient A—0.1% formic acid in water B—MeCN:water:MeOH (26:60:14), 0.1% formic acid Flow rate—0.175 mL min^{-1}	MS-ESI negative ion mode for folic acid and positive ion mode for 5-CH_3-H_4 folate w/SIM	%RSD—3.35	*J. Agric. Food Chem.*, 51, 1293, 2003[216]

Continued

Table 10.14 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
LC-MS (Continued)					
Foods/H_4 folate, 5-CH_3-H_4 folate, 5-CHO-H_4 folate	Add 40 mL 0.05 M HEPES/CHES buffer, pH 7.85 (containing 2% AA and 0.01 M 2-MCE, vortex, boiling water bath, 10 min. Cool. Apply trienzyme treatment to the sample suspensions. Clean up—SPE (SAX) cartridge	Aqua C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—gradient A—0.1% formic acid in water B—MeCN Flow rate—0.8 mL min^{-1}	MS/MS-ESI Positive ion mode w/SRM	DL—0.08–0.5 ng g^{-1} %Recovery—80–110	*Anal. Biochem.*, 315, 247, 2003;[217] *J. Chromatogr. B*, 792, 167, 2003;[218] *Anal. Bioanal. Chem.*, 376, 149, 2003;[219] *J. Food Compos. Anal.*, 17, 475, 2004[220]
Various foods/ 5-CH_3-H_4 folate, folic acid	Add 50 mL extraction buffer to the sample (infant formula—use Hex to remove lipid before extraction). Add 1 mL amylase (40 mg mL^{-1}), incubate, 37°C, 1h, add 1 mL protease (1 mg mL^{-1}), incubate, 37°C, 3 h. Heat in boil water, 90°C, 15 min, cool, add 0.2 mL conjugase, incubate, 37°C, overnight. Heat, 90°C, 15 min. Centrifuge, dilute the supernatant to 100 mL. Clean up—SPE (SAX) cartridge	For LC-MS/MS: Luna C_{18}, 5 µm, 15 cm × 4.6 mm Mobile phase—gradient A—0.1% formic acid in water B—MeCN-water-MeOH (26-60-14), 0.1% formic acid Flow rate—0.175 mL min^{-1} For LC only: Adsorbosphere C_{18}, 3 µm, 15 cm × 4.6 mm Mobile phase—gradient A—phosphate buffer, pH 2.2 B—MeCN Flow rate—1 mL min^{-1}	MS-ESI negative ion mode for folic acid and positive ion mode for 5-CH_3-H_4 folate or UV 280 nm 350 nm Fluorescence Ex λ = 290 Em λ = 355	From LC result: DL (µg 100 g^{-1}) 0.5–1.0 (UV) <0.1 (fluorescence) %Recovery—90–105	*J. Agric. Food Chem.*, 51, 3726, 2003;[221] J. *Agric. Food Chem.*, 51, 354, 2003[222]
Spinach/5-CH_3-H_4 folate, 5-CHO-H_4 folate, H_4 folate	Grind plant material to find powder under liquid N_2. Mix 0.5 g sample w/2.5 mL extraction buffer, heat at 100°C, 10 min. Cool, treat w/conjugase at 37°C, 4 h. Heat, 100°C, 10 min. Cool, centrifuge, filter.	Purospher Stan RP-18, 5 µm, 15 cm × 4.6 mm Mobile phase—gradient A—0.1% formic acid in water B—0.1% formic acid in MeCN Flow rate—1.0 mL min	MS/MS-ESI Positive ion mode w/MRM	DL—20–90 pg mL^{-1} QL—75–250 pg mL^{-1} %Recovery—89–128	*J. Chromatogr. A*, 1078, 59, 2005[223]

Green, red peppers/ folates	Homogenize sample w/phosphate buffer (0.1 M, pH 6.0). Digest sample w/α-amylase, protease and rat plasma conjugase. Clean up—Anion-exchange SPE	Luna C_{18}, 4 μm, 15 cm × 2.1 mm Mobile phase—gradient 0.1% formic acid in water—0.1 formic acid in MeCN Flow rate—0.2 mL min^{-1}	MS/MS-ESI Positive ion mode w/MRM	—	*J. Agric. Food Chem.*, 54, 9998, 2006[224]
Pharmaceuticals					
Multivitamin-mineral preparations/ folic acid	Add 100 mL extraction buffer (0.5 M NaAC-5% AA, 0.6% Na_2EDTA, 7% 2-MCE, pH 6) to 5 g ground sample, autoclave, 121°C, 5 min. Cool the supernatant quickly, dilute to 250 mL w/extraction buffer. Shake, centrifuge an aliquot 10 mL, 5°C. Filter	Ultrasphere ODS, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic 30 mM KH_2PO_4, pH 2.2: MeOH (78:22) Flow rate—1 mL min^{-1}	UV 281 nm	%Recovery—97	*J. Pharm. Biochem.* Anal., 23, 437, 2000[225]
LC-MS					
Multivitamin—multielement tablets/folic acid	Add 500 μL IS to 0.3 g homogenized sample, dilute the sample w/10 mL 0.1% dithiothreitol in MeOH:water (1:1) (pH 7.5). Vortex, mix using orbital rotator, 30 min. Centrifuge sample (pH 6.2), 10 min and filter	Supelco Discovery HS-F5, 5 μm, 15 cm × 4.6 mm Mobile phase—gradient 1% formic acid in water—1% formic acid in MeOH Flow rate—0.5 mL min^{-1}	MS/MS-ESI Positive ion mode w/MRM	DL (on-column) —0.02 ng QL (on-column) —0.06 ng %Recovery >95	*J. Chromatogr. A*, 1135, 203, 2006[226]
Biologicals					
Liver/Folic acid, H_4 folate, 5-CH_3-H_4 folate, 5-CHO-H_4 folate, 10-CHO-H_4 folate	Extract w/100°C buffer containing 2% AA, 0.2 M 2-MCE, 50 mM HEPES, 50 mM CHES, pH 7.5. Heat at 100°C, 10 min, homogenize, centrifuge. Recentrifuge supernatant, aspirate lipid layer, digest w/rat plasma conjugase, and filter	Ultrashpere I.P., 15 cm × 4.6 mm Mobile phase—gradient A—water B—water:95% EtOH (1:1) both A and B containing 5 mM TBAP and 5 mM 2-MCE Flow rate—1 mL min^{-1}	Microbiological *L. rhamnosus*	—	*Anal. Chem.*, 116, 393, 1981;[227] *Anal. Biochem.*, 142, 529, 1984;[228] *Proc. Natl. Acad. Sci. USA*, 80, 6500, 1983[133]

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Table 10.14 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Biologicals (Continued)					
Tissue, liver/ Polyglutamyl folates 1-7, H_2 folate 1-7, H_4 folate 1-7, 5-CH_3-H_4 folate 1-7, 5-CHO-H_4 folate 1-7, 10-CHO-H_4 folate 1-7	Liver—add 5–10 vol boiling extraction solution (2% AA, 10 mM 2-MCE in 0.1 M Bio-Tris, pH 7.8), boil 15 min. Homogenize, centrifuge. Store, evacuated, –70°C. Clean up—Folate binding protein-Sepharose 4B affinity column	Econosphere C_{18}, 5 μm, 10 cm × 4.6 mm Mobile phase—gradient A—5 mM TBAP, 0.5 mM DET in 25 mM phosphate/ Tris buffer, pH 7.4 or 25 mM NaCl in water B—5 mM TBAP, 0.5 mM DET in 25 mM phosphate/Tris buffer, pH 7.4 in MeCN: EtOH:water (64:9:27) Flow rate—1 mL min^{-1}	PDA 350 nm 10-CHO-H_4 folate 258 nm	—	*Anal. Biochem.*, 168, 247, 1988[229] & 182, 89, 1989;[230] *Cancer Res.*, 51, 16, 1991;[231] *J. Nutr. Biochem.*, 2, 44, 1991[232] & 3, 519, 1992;[233] *J. Nutr.*, 122, 986, 1992[234]
Liver/H_4 folate, 5-CH_3-H_4 folate, 5-CHO-H_4 folate	Homogenize in ice-cold 1% AA solution, flush w/N_2 and incubate overnight, 37°C. Centrifuge, adjust pH to 4.9. Boiling water bath, 60 min. Centrifuge, add 3 vol acetone to supernatant. Centrifuge, rotovap. Adjust volume to 50 mL. Clean up—SPE (Baker 10), Quaternary amine (N^+) strong anion-exchange	Spherisorb-ODS, 30 cm × 3.9 mm Mobile phase—isocratic MeCN:water containing 30 mM NaH_2PO_4 (10.5:89.5) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 300 Em λ = 360	DL (on-column) —0.4–2.0 pmol %Recovery— 95–123	*Anal. Biochem.*, 176, 406, 1989[235]
Plasma/5-CH_3-H_4 folate	Add 150 μL β-hydroxy ethyltheophyline (2 mg mL^{-1}) (IS) to 1.5 mL plasma. Bond Elute w/MeOH, evaporate and reconstitute w/mobile phase.	Nova-Pak, Phenyl cartridge, 4 μm, 10 cm × 8 mm Mobile phase—isocratic MeOH:0.05 M KH_2PO_4, pH 3.5 (15:85) Flow rate—2 mL min^{-1}	5-CH_3-H_4 folate EC +350 mV versus Ag/AgCl	%Recovery— 81–89	*Biomed. Chromatogr.*, 3, 58, 1989[236]

Serum, whole blood/5-CH_3-H_4 folate	Add 1.8 mL AA, 10 g L^{-1} to 0.4 mL whole blood, incubate 1 h, ambient. Add 0.2 mL 60% perchloric acid, centrifuge, mix 0.2 mL supernatant w/0.5 mL AA (30 g L^{-1}). Add 40 mg AA and 0.1 mL perchloric acid to 1 mL serum, centrifuge	Spherisorb S5 ODS, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:0.033 M orthophosphoric acid, pH 2.3 Flow rate—1.5 mL min^{-1}	Fluorescence Ex λ = 295 Em λ = 365	DL (on-column) —10 pg %Recovery Serum—84.7 Blood—86.3	*Metabolism*, 39, 902, 1990[237]
Plasma/H_4 folate, 5-CH_3-H_4 folate	Store, frozen w/addition of 1 mg mL^{-1} AA. Thaw, add equal volume 0.5 M perchloric acid, centrifuge, and filter	IRICA phenyl, 15 cm × 4.6 mm Mobile phase—isocratic MeCN:20 mM NaOAC, pH 3.6, containing 0.1 M EDTA (3.5:96.5)	EC +300 mV versus Ag/AgCl	QL—0.13–0.15 ng mL^{-1} %Recovery—77.6–83.0	*J. Vet. Med.* Sci., 54, 249, 1992[238]
Standards/Folic acid, H_2 folate, H_4 folate, 5-CH_3-H_4 folate, 5-CHO-H_4 folate	Dilute standards in 1% AA	Hypersil ODS, 3 µm, 25 cm × 4.6 mm Mobile phase—gradient A—5 mM KH_2PO_4 B—MeCN Flow rate—0.9 mL min^{-1}	UV 295 nm Fluorescence Ex λ = 295 Em λ = 356	DL (on-column)—1.1–2.4 ng (UV)	*J. Chromatogr.*, 540, 207, 1991[239]
Tissue, blood, erythrocytes, serum cerebro-spinal fluid foods/Folic acid, H_2 folate, H_4 folate, 5-CH_3-H_4 folate, 5-CH_3-H_2 folate, 5-CHO-H_4 folate, 5, 10-CH_2-H_4 folate	Erythrocytes: add 400 mL AA (20 g L^{-1}) to 200 mL blood, incubate 3 h, 25°C. Add 50 µL 11M perchloric acid, vortex. Neutralize w/11M KOH, centrifuge	Nova-Pak phenyl, 4 µm, 7.5 cm × 3.9 mm Mobile phase—isocratic MeOH:0.05 M KH_2PO_4, pH 3.5 (15:85), 25°C Flow rate—0.4 mL min^{-1}	EC +450 mV versus Ag/AgCl Fluorescence Ex λ = 295 Em λ = 365 5,10-CH_2-H_4 folate Ex λ = 360 Em λ = 470	DL (on-column)—0.3–0.52 ng (fluorescence)	*Food Chem.*, 47, 79, 1993[58] & 50, 307, 1994[240] &, 53, 329, 1995;[241] *Biochem. Mol. Med.*, 58, 93, 1996[242]

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Table 10.14 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Biologicals (*Continued*)					
Serum, urine/ 5-CH_3-H_4 folate, 5-CHO-H_4 folate	Serum—Add 1.5 mL MeCN to 1 mL sample, vortex and centrifuge. Extract MeCN supernatant w/7 mL $CHCl_3$, centrifuge, remove aqueous phase. Dilute w/5 mM TBAP, adjusted to pH 6.5. Urine—Dilute w/eluent A, SPE-Supelco C_{18}, elute w/2 mL 50% MeCN, extract MeCN w/$CHCl_3$, centrifuge, collect aqueous phase, dilute w/eluent A Clean up—column switching to direct analytes to analytical column from enrichment column	In-line Enrichment—C_{18} Macherey & Nagel, 5 µm, 3 cm × 4 mm Analytical—C_{18} Macherey % Nagel 3 µm, 25 cm × 2 mm Chiral—Resolvosil BSA-7 7 µm, 15 cm × 4.6 mm Eluent A—5 mM TBAP adjusted to pH 6.5 Eluent B—1.5 mM Na_2PO_4, 0.75 mM TBAP containing 7.5% IPA Eluent C—28 mM phosphate containing 6 mM sodium azide Eluent D—phosphoric acid	Fluorescence Ex λ = 360 Em λ = 470	—	*J. Chromatogr. B*, 669, 319, 1995[243]
Tissue, cells/H_4 folate, 5-CH_3-H_4 folate, 5, 10-CH_2-H_4 folate, folic acid	Add 2 mL extraction buffer (50 mM potassium tetraborate, 1% sodium ascorbate, pH 9.2) to sample, homo-genize (for cells sample, add 0.2% Triton x-100), transfer to boil-ing water, 15 min. Cool in ice. Adjust pH to 7, centrifuge, 4°C	FBP-Attiprep 10 affinity column to purify folate from extract. Betasil Phenyl, 25 cm × 4.6 mm Mobile phase—isocratic 28 mM K_2HPO_4—60 mM H_3PO_4 Flow rate—0.7 mL min^{-1}	Electro-chemical detection	$\%RSD_{intra}$—0.6–16 $\%RSD_{inter}$—5.2–13	*Clin. Chem.*, 46, 404, 2000[244]
Blood/5-CH_3-H_4 folate	Mix 0.1 mL whole-blood w/0.3 mL 57 mM AA, incubate, 37°C, 1h. Dilute w/0.6 mL buffer solution (0.2 M K_2HPO_4, 30 mM 2-MCE, pH 8.5). Heat, 100°C, 10 min, centrifuge	Zorbax StableBondSB C_{18}, 5 µm, 15 cm × 4.6 Mobile phase—isocratic 0.6% HAC:MeOH (86:14) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 295 Em λ = 360	%RSD—1.1–6.1 %Recovery—92–105	*J. Chromatogr. B*, 766, 331, 2002[245]
Human serum/ 5-CH_3-H_4 folate	Solid phase extraction SPE cartridge (Bond-Elut phenyl)	Luna C_{18}, 5 µm, 15 cm × 4.6 mm Mobile phase—isocratic MeCN:water:MeOH (26:60:14, 0.1% formic acid) Flow rate—0.2 mL min^{-1}	MS-ESI Positive ion mode w/SIM	%CV—5.3–7.6	*Anal. Biochem.*, 298, 299, 2001[246]

Human plasma/ folic acid, H_4 folate, 5-CH_3-H_4 folate, 5-CHO-H_4 folate	Add 2 mL MeCN to 2 mL spiked sample, vortex and centrifuge. Filter supernatant, rinse the precipitate w/MeCN and filter. Evaporate combined extract to dryness under vacuum, reconstitute in 100 μL MeCN containing 0.1% NH_4AC.	HILIC, 5 μm, 15 cm × 1.0 mm Mobile phase—isocratic MeCN:water (75:25, 5 mM NH_4AC) Flow rate—50 μL min^{-1}	MS/MS-ESI Negative ion mode w/MRM	DL—37.5–425 pM QL—360–1250 pM %RSD_{intra}—3.7–6.5	*Anal. Chem.*, 73, 5358, 2001[247]
Human plasma, serum/Folic acid, H_2 folate, H_4 folate, 5-CH_3-H_4 folate, 5-CHO-H_4 folate	Solid phase extraction Sep-Pak C_{18} cartridge Or Solid phase affinity extraction IS—^{13C5}5- CH_3-H_4 folate	Nova-Pak Phenyl, 4 μm, 15 cm × 3.9 mm Mobile phase—gradient A—25 mM NH_4COOH adjust pH to 3.5 w/formic acid B—25 mM NH_4COOH in MeOH Flow rate—0.35 mL min^{-1}	MS-ESI Positive ion mode w/SIM	QL—0.39 ng mL^{-1} %Recovery—98	*J. Chromatogr. B*, 765, 141, 2001;[248] *Anal. Biochem.*, 313, 117, 2003[249] & 325, 41, 2004[250]
Human blood, urine/5-CH_3-H_4 folate	Urine—mix 55 mL sample w/5.5 mL potassium phosphate buffer, 0.5 M, pH 7, stand, room temperature, 30 min, filter. Plasma—mix 5 mL sample w/20 mL 0.1 M K_2HPO_4, pH 7 containing 0.05 M sodium ascorbate and 0.01 M 2-MCE, flash w/N_2, boiling water, 10 min. Cool, centrifuge, mix the pellet w/15 mL extract buffer, centrifuge, combine two supernatants. Clean up—Solid phase affinity extraction IS—$^2H_4$ folate	Superspher 100 RP 18, 4 μm, 25 cm × 2 mm Mobile phase—isocratic 0.1 M HAC (adjust pH to 3.3 w/NH_4AC):MeCN (90:10) Flow rate—0.25 mL min^{-1}	MS-ESI Negative ion mode w/SIM	DL—0.2 nM QL—0.55 nM %CV—7.4	*Anal. Biochem.*, 305, 206, 2002[251]
Human serum, Erythrocytes/ 5-CH_3-H_4 folate, 5-CHO-H_4 folate, H_4 folate	Mix 275 μL sample w/770 μL SPE sample buffer (1% ammonium formate, 0.1% AA, pH 3.2), incubate, 4°C, 20 min. IS - ^{13}C folate Clean up—SPE (Phenyl)	Luna C_8, 5 μm, 15 cm × 3 mm Mobile phase—isocratic MeOH:MeCN:HAC (40:10:1) Flow rate—0.25 mL min^{-1}	MS/MS -ESI Positive ion mode w/MRM	%Recovery—67–83	*Clin. Chem.*, 50, 423, 2004[252] & 51, 2318, 2005[253]

Continued

Table 10.14 (Continued)

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
LC-MS (*Continued*)					
Plasma/5-CH_3-H_4 folate, folic acid	Mix 2 mL sample w/10 mL extract buffer (0.1 M K_2HPO_4, pH 7 containing 0.05 M AA and 0.01 M 2-MCE), flash w/N_2, boiling water, 20 min. Cool, centrifuge, mix the pellet w/10 mL extract buffer, centrifuge, combine two supernatants. Clean up—Solid phase affinity extraction	Xterra MS C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic 10 mM formic acid (pH 2.3): MeCN (90:10) Flow rate—1 mL min^{-1}	MS/MS -ESI Positive ion mode w/MRM	DL (pM) Folic acid—500 5-CH_3-H_4 folate—12	*Anal. Biochem.*, 326, 129, 2004[254]
Human serum, plasma/folic acid, 5-CH_3-H_4 folate	Spike 500 µL sample (pH 7.2) w/72 µL 10% dithiothreitol, stand, 25°C, in the dark, 15 min. Add 500 µL 10% AA, vortex, stand on ice, 15 min. Clean up—SPE C_{18}	Supelcosil LC-CN cyano, 5 µm, 25 cm × 4.6 mm Mobile phase—gradient A—0.4% formic acid in water B—0.4% formic acid in MeOH Flow rate—0.75 mL min^{-1}	MS/MS -ESI Positive ion mode w/MRM	QL—0.14 nM for folic acid %RSD—0.7–10.4	*Anal. Chem.*, 77, 3586, 2005[255]
Whole blood/folic acid, 5-CH_3-H_4 folate, 5-CHO-H_4 folate	Add 10 µL IS in IPA and 100 µL 12.1 N HCl to 100 µL sample, fill headspace w/argon gas and seal. Heat the mixture at 110°C, 4 h. Cool, dilute w/200 µL water. Clean up—SPE C_{18}	Synergi 4 µm Hydro-RP, 15 cm × 4.6 mm Mobile phase—isocratic MeCN:water (70:30) Flow rate—0.5 mL min^{-1} Add 0.025% formic acid at 0.2 mL min^{-1} prior to entry into the source	MS/MS-APCI Positive ion mode w/MRM	QL—56.6 nM folate L^{-1} red blood cell	*J. Agric. Food Chem.*, 53, 7390, 2005[256]
Erythrocytes/folic acid, 5-CH_3-H_4 folate, 5-CHO-H_4 folate, H_4 folate, H_2 folate, 5,10-CH_2-H_4 folate, 5-CHO-H_4 folate, 10-CHO-H_4 folate	Extract folates using affinity chromatography.	Xterra MS C_{18}, 3.5 µm, 10 cm × 3.9 mm Mobile phase—isocratic 10 mM formic acid:MeCN (90:10) Flow rate—0.8 mL min^{-1} The column was connected to MS by a splitter with a split ratio of 1:5	MS/MS-ESI Positive ion mode w/MRM	QL—0.4 nM L^{-1} %Recovery—97–107	*Clin. Chem. Lab. Med.*, 44, 450, 2006[257]

most effective under acid conditions, and dithiothreitol is most effective at neutral pH. Antioxidants convert 5-methyldihydrofolates to reduced forms. 5-Methyltetrahydrofolic acid is stabilized at pH 7.3 and pH 9.0 by dithiothreitol, but not at pH 3.5.[58] For quantitation of 10-formyltetrahydrofolate, the Wilson and Horne buffer at pH 7.85 is useful, preventing thermal conversion of the 10-formyl form to 5-formyltetrahydrofolate.[48] In addition, 5-formyltetrahydrofolate is unstable under acidic conditions.[64] Konings[206] and Konings et al.[207] used CHES-HEPES buffer containing ascorbic acid and 2-mercaptoethanol to stabilize folic acid and folates in a validated LC assay developed at the Interspectorate for Health Protection, The Netherlands. Finglas et al.,[258] in an effort to standardize LC methodology for assay of naturally occurring folates in food, compared data from nine laboratories from six different countries. Recommendations for stabilization included use of ascorbic acid containing buffers and nitrogen flushing as significant factors. This study completed from 1990 to 1997 added much insight into problems presented by LC analysis of natural products. Further, the study showed the need for routine use of standard reference materials. Such materials included were CRM 121, wholemeal flour; CRM 421, milk powder; CRM 485, mixed vegetables; and CRM 487, pig's liver. Kall et al.[225] optimized the extraction of folic acid from multivitamin preparations containing minerals through use of 0.5 Mm sodium acetate containing 5% ascorbic acid, 0.7 mL L^{-1} 2-mercaptoethanol, and 6 g L^{-1} Na_2 ETDA. The extraction solution prevented degradation of folic acid for over 24 h when samples were refrigerated and protected from light.

2. *Destruction of Sample Matrix and Release of Folates*: In order to facilitate later determinative steps and ensure complete folate extraction, biological samples are usually heated at 100°C or autoclaved to precipitate proteins and inactive enzymes that catalyze folate oxidation or interconversion. In addition, enzyme digestion by α-amylase and protease is now becoming accepted as necessary to completely free nonspecifically bound folates (Section 10.5.1). Thermal treatment helps to free folate from specific folate-binding proteins. Extraction of serum includes protein removal by perchloric acid, heating, or addition of organic solvents (acetonitrile, acetone).[20] Various extraction approaches for serum are provided in procedures summarized in Table 10.14. Solid phase extraction (SPE) is finding increased use as an integral step in serum extractions. Urine can be diluted, treated by SPE, and directly injected.[243] Extensive studies in Finland by Vahteristo and colleagues[59,199–204,208] on the LC quantitation of folates in food use SPE cleanup by strong anion exchange following deconjugation by hog kidney conjugase. The extracts are relatively free of chromatographic interference. Several cleanup procedures using SPE are available in the literature. Affinity chromatography, discussed later in text, is an excellent concentration and cleanup approach before LC analysis of folate; however, the Finnish procedure is simpler to implement and useful for many matrices. Table 10.14 gives specific citations.
3. *Deconjugation*: Depending on the objectives of the study, the analyst has the choice of determining folates as the monoglutamylfolates or quantifying the folates as the γ-glutamyl polymers. If the polymeric forms are to be chromatographed, all indigenous conjugase enzymes must be quickly inactivated by heating. Older literature states that rapid heating to 70°C inactivates conjugases.[48,201] Although most LC methods are based on resolution of monoglutamylfolates, excellent LC methods are available to resolve the γ-glutamyl polymeric folates. Inclusion of a conjugase digestion is necessary to convert the γ-glutamyl polymeric folates to the monoglutamate form. Differing from conjugase treatments for microbiological assay, an enzyme must be used that converts the polymeric forms only to the monoglutamate level.

Hog kidney and rat plasma conjugase treatment is common. Chicken pancreas is not suitable for methods requiring only monoglutamylfolates in the extract because it yields a mixture of mono- and diglutamate.[132]

4. *Affinity Chromatography*: Affinity chromatography developed into an integral part of sample preparation procedures for food, serum, and other biologicals. The methodology was developed by Selhub and colleagues and applied to various matrices.[193,194,229–234,244,259] The procedure for isolation of the bovine milk folate-binding protein was detailed by Selhub et al.[229,230] Selhub's work, since the early 1980s, has provided a significant tool that allows folates in biological extracts to be purified and concentrated extensively before chromatography. Further, the extract is clean enough to allow use of photodiode array (PDA) detectors. Affinity chromatography was integrated into the LC analysis of cereal grain products by Pfeiffer et al.[137] Pfeiffer's procedure incorporates several procedural steps developed by various researchers over the last decade. The method is provided in Section 10.6. Anyone considering the use of LC for folate analysis should become familiar with the application of affinity chromatography to the sample preparation protocol. Bagley and Selhub[259] provide an in-depth, procedural guide to the combined use of affinity chromatography and LC for folate assay from biologicals.

10.4.3.1.2 Chromatography parameters

10.4.3.1.2.1 Supports and mobile phases. Monoglutamylfolates can be resolved by ion-exchange or reversed-phase chromatography. Ion exchange on several supports using open-column chromatography provided the first chromatographic resolution of folates. Quantification during this time period (1960–1970) relied on microbiological assay of the column eluents. Mullin and Duch,[260] in their 1992 review, showed that most LC methods to that time relied on C18 reversed-phase supports and, more infrequently, use of phenyl supports. Later procedures (Table 10.14) have in most cases used reversed-phase chromatography with C18. When ion-pairing is used, the reagent has been predominantly tetrabutylammonium phosphate (TBAP) or tetra-*n*-butylammonium hydrogen sulfate (TBAHS). Ion-pair reversed-phase mobile phases are not suitable for more recent LC-MS assays since the ion-pair such as TBAP are not volatile.[117] The review by Quinlivar et al.[117] provides valuable comparisons of detector compatibilities with reversed-phase and ion-pair reversed phase systems. Most systems require gradient elution. Mobile phase systems are given in Table 10.14 for recently published procedures. Figure 10.5 is an example of chromatography suitable for resolution of polyglutamylfolates and monoglutamyl forms. Descriptions in Table 10.15 give parameters for the ion-pair system used by Selhub's group in conjunction with affinity chromatography cleanup and PDA detection.

10.4.3.1.2.2 Detection. Ultraviolet (UV) absorbance, fluorescence, and EC detection can be used effectively for folate quantitation. Microbiological assay of column eluents were used in earlier studies before the availability of more effective LC detectors. Lucock et al.[241] optimized detection parameters for UV, fluorescence, and EC detection of 11 monoglutamylfolates. Ultraviolet spectra were obtained by PDA. Data provided by Lucock et al.[241] is highly useful for anyone initiating LC analysis of folates (Tables 10.15 and 10.16).

As mentioned previously, the need for more highly specific and sensitive methods for the analysis of folates and folic acid in both food and clinical laboratories led to the rapid advancement of LC-MS-based methods when instrumentation became available over the last decade. Since 2001, many excellent LC-MS or LC-MS/MS methods based on stable isotope dilution have been introduced, most with well-defined performance parameters.[215,217,226,246,248,249,251–253,255–257,220] Characteristic of the newer procedures for folate analysis is the work of Freisleben et al.[197] and Rychlik.[220] Their methodology demonstrates the

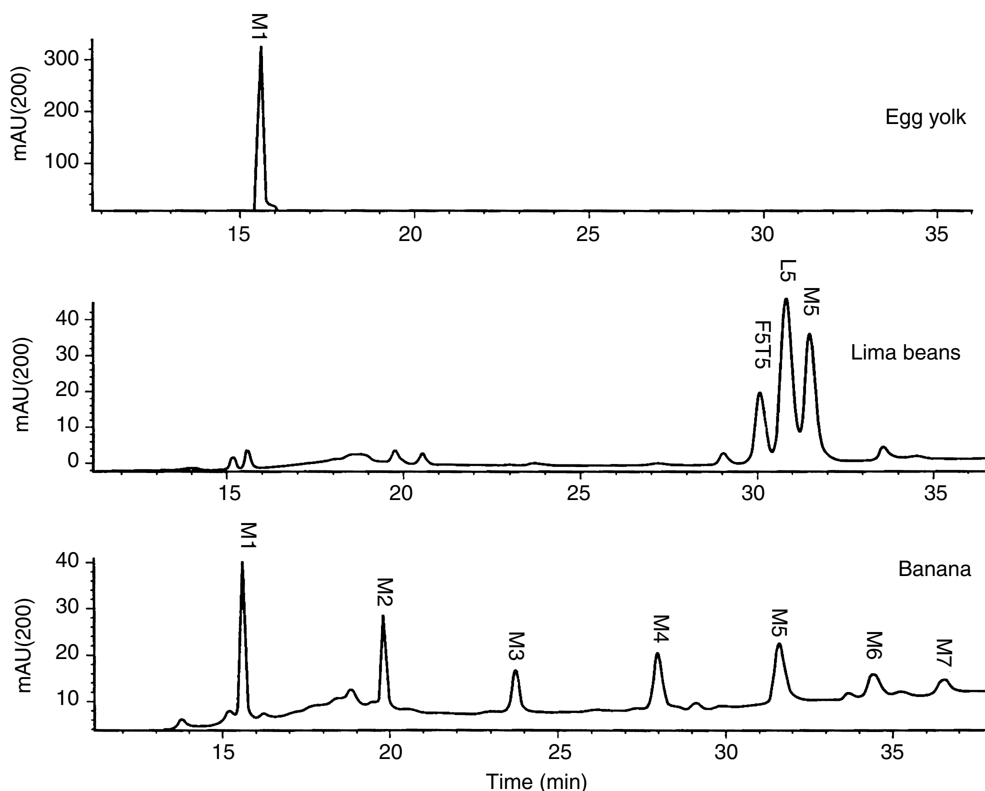

Figure 10.5 Ion-pair LC of purified food folates chromatography. F = 10-CHO-H_4 folate, T = O unsubstituted tetrahydrofolates, L = 5-CHO-H_4 folates, M = 5-CH_3-H_4 folates. Numbers following letters indicate number of glutamate residues. (Reproduced from Seyoum, E. and Selhub, J., *J. Nutr. Biochem.*, 4, 488, 1993. With permission.)

superiority of stable isotope dilution with LC-MS/MS compared to other approaches not employing stable isotopes or mass spectrometry. LC-MS/MS assay of various foods showed better selectivity and precision compared to analysis by LC with fluorometric detection.[217] Use of stable isotopes improves accuracy by correcting for extraction losses and variations in ionization efficiencies.[220] In general, stable isotopes when used as internal standards can simplify and speed up extraction procedures.[246] Figure 10.6 shows an LC-MS/MS chromatogram using selected ion monitoring (SRM) of a raw spinach sample compared to the UV chromatogram.[220]

Pfeiffer et al.[252] introduced an LC-MS/MS with isotope dilution for the analysis of 5-methyl-H_4 folate, 5-formyl-H_4 folate, and folic acid in human serum as a candidate reference method for folate assay of serum. Excellent accuracy, precision, and sensitivity indicated advantages over currently accepted microbiological and radioassay methods.

10.4.3.1.2.3 Internal standards. Owing to the complexity and varying responses of folates to different detection modes, internal standards have not been routinely applied in LC methods. [3H] folic acid was effectively used as an internal standard (IS) for folate analysis.[261] The IS was used in conjunction with a *L. casei* ssp. *rhamnosus* assay of LC resolved 5-methyl-H_4 folate. Use of a microplate assay allowed effective quantitation of the 5-methyl-H_4 folate. The tritiated IS was quantified by scintillation counting. A detection limit of 1 ng/mL was obtained and recoveries approximated 100%. 13C-labeled folic acid, [$13C_5$]-folic acid, [$13C_5$]-5-CH_3-H_4 folate, [$13C_5$]-5,10-CH=H_4 folate, [$2H_4$]-folic acid,

Table 10.15 Physico-Chemical Data Useful for the LC Analysis of Monoglutamylfolates[a]

Folymono-glutamate	Chromatographic retention time (min) at 0.4 mL min^{-1}[b]	λ_{max} (nm) for UV detection between 240 and 400 nm [relative absorptivity (%) is given in parentheses]	Optimum voltage for electrochemical detection using a glassy carbon electrode (mV)	Optimum emission wavelength (nm) at an excitation wavelength of 295 nm[c]	Purity angle purity threshold value <1.0 peak implies peak homogeneity
P-ABG	2.82	272 (100)	900	(1) 358	
				(2) 609	0.23
$5CH_3$-H_3PteGlu	4.32	276 (100)	No signal below 1000	607	0.10
H_4PteGlu	4.06	267 (100)			
		290 (7)	400	358	0.58
$5CH_3$-H_4PteGlu	6.16	267 (100)		(1) 358	
		290 (99)	400	(2) 607	0.86
5CHO-H_4PteGlu	9.75	285 (100)	600	(1) 372	
				(2) 607	0.86
5,10CHH_4PteGlu	11.43	(1) 355 (100)			
		(2) 255 (36)	900	607[b]	0.08
		(3) 280 (26)			
H_2PteGlu	12.79	(1) 280 (100)			
		(2) 300 (82)	400	607	0.04
		(3) 325-350 (27-46)			
PteGlu	14.31	(1) 280 (100)[d]		No emission in the	
		(2) 300 (82)	850	350–365 region	0.06
		(3) 350 (25)			
5,10CH_2H_4PteGlu	21–26	300 (100)	500	(1) 363	0.01
				(2) 607	

[a] Reproduced with permission from Reference 241.
[b] Mobile phase—15% (v/v) methanol in 0.05 M KH_2PO_4, pH 3.5.
[c] Excitation λ is not optimum.
[d] Spectral characteristics of $PteGlu_3$ are almost identical to PteGlu.

Table 10.16 Comparison of UV, Fluorescence, and Electrochemical Detection Sensitivity for Monoglutamylfolates and Related Compounds[a]

	Amount on column, chromatographic conditions as described[e]				
	Minimum UV detection limit equivalent to	Minimum electrochemical detection limit		Minimum fluorescence detection limit at 295 nm excitation/365 nm	
Congener	0.0003 au	450 mV	800 mV	emission	Common reason for measurement
p-ABG	530 pg	[b]	[b]	3.12 ng	Stability studies on C9-N10 cleavage
$5CH_3H_2$PteGlu	1.2 ng	[b]	[b]	[c]	Stability studies, $5CH_3$-H_4PteGlu purity analysis
H_4PteGlu	790 pg	1.1 ng	440 pg	4.9 ng	Biochemical studies
$5CH_3$-H_4PteGlu	1.7 ng	300 pg	240 pg	300 pg	Food, plasma, whole blood, and pharmaceutical anlaysis; can be used for methotrexate rescue
$PteGlu_3$	2.3 ng	[d]	—	—	Synthetic substrate useful in biochemical studies
$5CHOH_4$PteGlu	3.8 ng	No signal	800 pg	18.8 ng	Plasma and pharmaceutical analysis in relation to methotrexate rescue; is a precursor for $10CHOH_4$PteGlu
$5,10CHH_4$PteGlu	7.4 ng (280 nm) 2.4 ng (355 nm)	[b]	[b]	[c]	Precursor for synthesis of $10CHOH_4$PteGlu
H_2PteGlu	2.7 ng	1.7 ng	180 pg	[c]	Biochemical studies
PteGlu	3.3 ng	[b]	[b]	[c]	Found in food and plasma following supplementation; pharmaceutical analysis
Aminopterin	2.64 ng	—	—	—	Antifolate
$5,10CH_2H_4$PteGlu	9.6 ng	2 ng	—	52.4 ng	Biochemical studies

[a] Table reproduced with permission from Reference 241.
[b] Oxidation state or occurrence makes this form of detection inappropriate.
[c] Inappropriate form of detection or excitation wavelength.
[d] Not studied.
[e] Mobile phase — 15% (v/v) methanol in 0.05 M KH_2PO_4, pH 3.5.

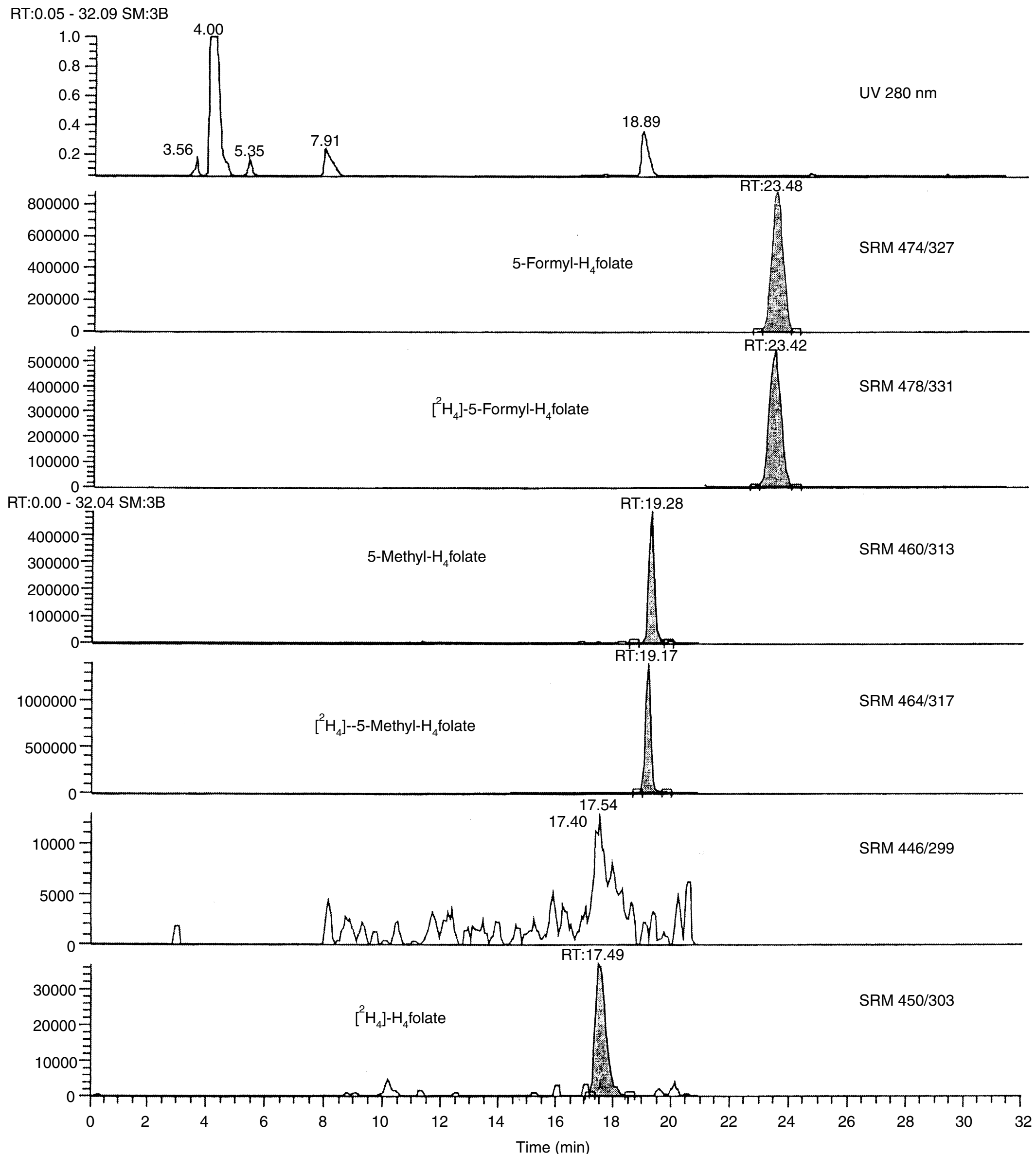

Figure 10.6 LC-MS/MS of a raw spinach sample obtained by selected ion monitoring (SRM) compared to the UV chromatogram. (Reproduced from Rychlik, M., *J. Food Compos. Anal.*, 17, 475, 2004. With permission.)

[$2H_4$]-tetrahydrofolic acid, [2H4]-5-methyltetrahydrofolic acid, [2H4]-5-formyltetrahydrofolic acid, and [2H4]-10-formylfolic acid have been used as stable-isotope internal standards in LC-MS methods.

10.4.3.2 *Optical biosensor-based immunoassays*

Several studies applied an optical biosensor-based immunoassay marketed by Biacore AB, Victoria, Australia and Uppsala, Sweden for assay of folate and other water-soluble vitamins.[262–264] The system entails biomolecular interaction analysis with an immunosensor

chip immobilized with the vitamin on a carboxymethyldextran surface. Detection is by surface plasmon response due to a refractive index change caused by ligand binding. The Biacore instrument provides real-time measurement, with no requirements for enzymes or isotopes and high precision.[262] For assay of folic acid in fortified foods, accuracy and precision were excellent and results compared closely to microbiological assay by *L. casei* ssp. *rhamnosus*.[262,263] Caselunghe and Lindeberg[263] reported that advantages of the method included fast sample preparation and low demands on time. A sample analysis run could be completed within 12 h for 40 sample extracts.

Indyk and Filonzi[265] expanded the Biacore instrument to assay of folate-binding protein in milk. Folate-binding protein was measured from its specific interaction with folic acid immobilized on the sensor surface through amine coupling with the glutamate-carboxyl group. Sample preparation for milk, colostrum, and milk powders only required dilution with buffer.

10.4.3.3 Status of folic acid and folate analysis

Folic acid and folate analysis is still conducted worldwide by a large and variable number of procedures with the acceptance of AACC Method 86–47 and AOAC Method 2004.05 that specifies the trienzyme procedures for liberation of folate from cereal matrices, more standardization of food extraction procedures might occur. It should be understood that the trienzyme procedure as originally proposed by De Souza and Eitenmiller and supported by Tamura is applicable to most foods. However, generalizations about applicability of a specific extraction procedure to different complex matrices should not be made. An often overlooked aspect of vitamin analysis has been lack of validation of the extraction with subsequent collection of poor, nonrepresentative data. This result can easily occur with folate assay. Use of LC and LC-MS methods are a great advance. However, recently published performance data of international laboratories including 26 laboratories (20 microbiological assay, four LC-UV, one LC-MS, one RPBA) showed that variation among laboratories was quite high.[266] Analysis of soybean flour, fish powder, and breakfast cereal ranged from 25% to 35%. Such comparative data shows the need for methodology standardization on an international basis.

10.5 Method protocols

Trienzyme Extraction, AOAC Official Method 2004.05[122,124]

Principle

A broad specificity protease (usually Pronase) and α-amylase are used to digest the food to release folate bound in the matrix. γ-Glutamyl hydrolase (conjugase) digestion reduces polyglutamyl folates to ≤3.

Procedure

1. To 0.25–1.0 g (db) sample, add 20 mL phosphate buffer (1.48 g dibasic sodium phosphate + 1 g ascorbic acid per 100 mL), pH 7.8. A 125 mL Erlenmeyer is often used, although test tubes of sufficient size can be used as well.
2. Dilute to 50 mL with water. Add 0.1–1.0 mL octanol as an antifoam agent and autoclave 15 min at 121°C.
3. Cool quickly and add 10 mL phosphate buffer. Add 1 mL protease solution (2 mg mL^{-1}, Pronase in water).
4. Incubate 3 h at 37°C.
5. Inactivate enzyme by placing flask in boiling water bath for 3 min or autoclaving. Cool.

6. Add 1 mL α-amylase solution (20 mg mL^{-1} in water). Incubate 2 h at 37°C.
7. Add 4 mL conjugase solution (chicken pancreas, 5 mg mL^{-1} in phosphate buffer, pH 7.8, prepared as directed). Incubate 16 h at 37°C.
8. Inactivate enzymes by placing flask in boiling water bath or autoclaving for 3 min. Cool.
9. Adjust extracts to pH 4.5 with HCl (1 + 1), dilute to 100 mL with water, filter.
10. Dilute extract aliquotes with equal volume of phosphate buffer, pH 6.8. Bring to known volume with water.

Determination of Folate in Cereal-Grain Food Products Using Trienzyme Extraction and Combined Affinity and Reversed-Phase Liquid Chromatography

J. Agric. Food Chem., 45, 407, 1997[137]

Principle

Extract folates by digestion with rat plasma conjugase, α-amylase, and protease in HEPES/CHES buffer, pH 7.85. The extract is purified by affinity chromatography with immobilized folate-binding protein. Folates are resolved by reversed-phase gradient LC and quantitated with UV diode array detection.

Chemicals and Materials

- HEPES/CHES buffer
- Affigel 10
- Isolated folate binding protein (FBP)
- 5-Methyltetrahydrofolate
- 5-Formyltetrahydrofolate
- Folic Acid
- 10-Formylfolic acid
- 5,10-methenyltetrahydrofolate HCl
- Pteroyltriglutamate
- Sodium ascorbate
- Dithiothreitol
- 2-Mercaptoethanol
- Trifluoroacetic acid

Enzymes

- Rat plasma conjugase
- α-Amylase
- Protease

Apparatus

- Polytron homogenizer
- Liquid chromatograph
- Diode array detector
- Glass columns (0.7 cm × 20 cm)

Procedure

Trienzyme Extraction

- Add 10 volumes HEPES/CHES buffer, pH 7.85 containing 2% sodium ascorbate and 10 mM 2-mercaptoethanol to 2 g dry sample.

- Boiling water bath, 10 min.
- Cool.
- Homogenize, Polytron.
- Digest with rat plasma conjugase and α -amylase, 4 h, 37°C.
- Digest with protease, 1 h, 37°C.
- Boiling water bath, 5 min.
- Cool.
- Centrifuge.
- Resuspend residue in extraction buffer.
- Centrifuge and combine supernatants.
- Filter, Whatman #1.
- Flush with N_2, store at 4°C.

Extract Purification

- Prepare affinity chromatography sorbent:FBP coupled to Affigel 10, 0.7 cm × 20 cm glass columns.
- Apply sample extract to affinity column equilibrated with 0.1 M potassium phosphate buffer, pH 7.0.
- Wash with 5 mL 0.25 M potassium phosphate, pH 7.0, containing 1.0 M NaCl.
- Follow with 5 mL 0.025 M potassium phosphate, pH 7.0.
- Elute folate with 2 mL 0.02 M trifluoroacetic acid/0.01 M dithiothreitol (discard) followed by 5.0 mL of 0.02 M trifluoroacetic acid/0.01 M. dithiothreitol (contains all folates).
- Add 30 mL 1 M piperazine.
- Add sodium ascorbate to 0.02% v/v.
- Add 2-mercaptoethanol to 10 mM.
- Flush with $N_{2.}$
- Store frozen.

Chromatography

Column	25 cm × 4.6 mm
Stationary phase	Ultramex C_{18}, 5 µm
Mobile phase	Gradient
	A—MeCN
	B—0.033 M phosphoric acid, pH 2.3
	0–8 min, 5% A, 95% B
	8–33 min, linear to 17.5% A
Column temperature	Ambient
Detection	Diode array, 280 nm
Calculation	Peak area
	External standard, peak area, least squares regression

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chapter eleven

Vitamin B_{12}

11.1 Review

Vitamin B_{12} deficiency, or pernicious anemia, was identified in 1824 with clinical symptoms being described in 1855.[1] It was not until 1925 that treatment protocols became apparent through the discovery by Whipple and Robscheit-Robbins that raw liver had curative effects for anemic dogs.[2] In 1926, Minot and Murphy reported that raw liver cured pernicious anemia in humans. That same decade (1927), Castle suggested the presence of the extrinsic factor in food and the intrinsic factor in the gastrointestinal system as the preventative factors for pernicious anemia.[1] In 1934, Whipple, Minot, and Murphy were awarded the Nobel Prize for Medicine for advances made in the treatment of pernicious anemia. Significant events in the more recent history of vitamin B_{12} include the isolation and naming of vitamin B_{12} and the first use of injections of the vitamin to cure deficiency (1948), the isolation of crystalline forms and identification of cyanocobalamin (CNCbl) and hydroxocobalamin (OHCbl) (1949), establishment of the structure of vitamin B_{12} and its microbial synthesis (1955), and chemical synthesis (1973).[1] Dorothy Hodgkin was awarded the Nobel Prize in 1964 for her work with x-ray crystallography to determine the structure.

Absorption of vitamin B_{12} requires enzyme and acid hydrolysis in the stomach to liberate cobalamin (Cbl) from macromolecules, primarily protein. After digestive release, Cbl binds to salivary proteins (R-binders or haptocorrins), and is subsequently bound by the intrinsic factor secreted by gastric parietal cells.[3] The intrinsic factor—vitamin B_{12} complex—then binds to receptors in the ileal mucosa and absorption occurs in the ileum through binding of Cbl in the presence of calcium with cell surface receptors and release of the intrinsic factor. After absorption, all circulating vitamin B_{12} is complexed with B_{12} plasma-binding proteins (transcobalamin I, II, or III) to form holoTCI, II, or III or with the B_{12} storage protein (haptocorrin) to form holohaptocorrin. Transcobalamin and haptocorrin complexes contain 20% and 80%, respectively, of total serum vitamin B_{12}.[3]

Pernicious anemia evolves through abnormal absorption of vitamin B_{12} resulting from inadequate digestion, lack of necessary binding factors including Ca^{2+}, lack of intrinsic factor due to lack of synthesis or autoimmune inactivation, absence of the delivery or storage proteins, and various other pathological states. Lack of sufficient dietary intake is usually not the cause of deficiency.[4,5,6] Malabsorptive disorders cause about 50% of preclinical cases.[6]

Effects of vitamin B_{12} deficiency include hematological, neurological, and gastrointestinal symptoms.[4] Hematological symptoms commonly referred to as pernicious anemia include megaloblastic anemia with diminished energy, fatigue, shortness of breath, and heart palpitations.[4,5] Etiology is similar to megaloblastic anemia associated with folate deficiency and arises from interferences with DNA synthesis. Since folate supplementation can

mask hematological changes due to lack of vitamin B_{12} but not reverse neurological damage associated with the deficiency, conservative approaches have been used in the initiation of folic acid supplementation programs (see Chapter 10). Hematological effects can be reversed by vitamin B_{12} treatment.[4]

Neurological effects include tingling and numbness in the arms and legs, motor disturbances (gait), and various cognitive changes (loss of memory, disorientation, dementia, and mood changes). Other effects include visual disturbances, insomnia, impotency, and impaired bowel and bladder control. Neurological effects can be reversed by treatment, but reversal depends on duration and extent of the neurological damage.[4]

Gastrointestinal effects include appetite loss, sore tongue, flatulence, and constipation.[4]

Total serum vitamin B_{12} measurement is used to screen for deficiency.[5] Levels of <59 pmol L^{-1} indicate deficiency. Levels of 100–150 pmol L^{-1} are considered moderately low. Such levels occur with folate deficiency and pose difficulty with interpretation.[5,6] For this reason, measurement of methylmalonic acid or homocysteine in the serum is recommended if vitamin B_{12} levels are <225 pmol L^{-1}. Methylmalonic acid concentrations rise when vitamin B_{12} levels are low. Homocysteine serum levels commonly rise if folate status is low (see Chapter 10). However, the Dietary Reference Intake (DRI) committee considering vitamin B_{12} did not consider homocysteine levels as a good indicator to differentiate vitamin B_{12} and folate deficiencies.[4] For adults, the DRI values were set on the basis of hematological status and serum vitamin B_{12} levels. A normal serum B_{12} level was assumed to be ≥150 pmol L^{-1} (200 pg 100 g^{-1}).[4] Indicators of hematological status included hemoglobin levels, hematocrit, erythrocyte count, mean cell volume, and reticulocyte number. Hebert[3] summarized sequential stages of vitamin B_{12} status and the use of various indicators to assess human status. Low serum holoTCII is the earliest indicator of negative vitamin B_{12} balance, which indicates inadequate delivery of vitamin B_{12} to DNA synthesizing cells. Serum holotranscobalamin (holoTCII) falls to low levels before total serum vitamin B_{12} lowers or deficiency occurs. Gibson[5] states that holoTCII concentration quickly decreases when vitamin B_{12} absorption is impaired; however, difficulty in measurement impedes the use of this marker for clinical use. Measurement of serum and erythrocyte concentrations remain common biochemical tests for status assessment; however, such measurements are often difficult to interpret.

All vitamin B_{12} found in the human diet is derived from microbial synthesis. Food sources include fermented foods and animal products. However, substantial amounts naturally occur only in animal products.[7,8] Low, but measurable, levels of vitamin B_{12} can sometimes be found in plant products owing to the presence of bacteria. In the animal kingdom, vitamin B_{12} is derived from the animal's diet or from synthesis by the gut microflora. The richest human food sources include meats, seafoods, eggs, and dairy products. Vegetarian diets can produce deficiency; however, most "true" vegetarians are aware of their need to supplement the vegetarian diet with vitamin B_{12}.[6–9] For vegetarians, 1–5 µg, orally, is sufficient. Therapeutic treatment of pernicious anemia requires 100 µg of vitamin B_{12} parenterally per month.[10] Data from the USDA National Nutrient Database for Standard Reference, Release 19 is given in Table 11.1.[11] Significant sources are quite limited to muscle foods and fortified products such as breakfast cereals. OHCbl is retained at higher levels than CNCbl.[1] Recommended Dietary Allowances (RDA) are 2.4 mg for adults, increasing to 2.6 µg during pregnancy, and 2.8 mg during lactation (Table 11.2).[4] The Reference Daily Intake (RDI) specified by the Nutritional Labeling and Education Act of 1990 is 6 µg.[12]

The primary metabolically active coenzyme form of Cbl is 5′-deoxyadenosyl cobalamin (AdoCbl). The AdoCbl-dependent enzymes catalyze intramolecular 1,2-rearrangements in which a group X and a hydrogen on adjacent carbon atoms exchange places.[13] The nature of the migrating group and the nature of the substituent on the carbon atom to which the group migrates segregates the enzymes into three classes (Table 11.3). The Class I enzymes

Table 11.1 Vitamin B_{12} Content of Various Foods[a]

Description	NDB no.	μg 100 g^{-1}	Description	NDB no.	μg 100 g^{-1}
Mollusks, clam, mixed species, canned, drained solids	15160	98.89	Salami, cooked, beef and pork	07069	3.65
Beef, variety meats and by-products, liver, cooked, pan-fried	13327	83.13	Fish, salmon, Chinook, smoked	15077	3.26
Turkey, all classes, giblets, cooked, simmered, some giblet fat	05172	33.25	Crustaceans, lobster, northern, cooked, moist heat	15148	3.11
Cereals ready-to-eat, GENERAL MILLS, Whole Grain TOTAL	08077	21.40	Fish, tuna, lights, canned in water, drained solids	15121	2.99
Braunschweiger (a liver sausage), pork	07014	20.09	Beef, rib, whole (ribs 6-12), separable lean only, trimmed to 1/4″ fat, all grades, cooked, roasted	13085	2.91
Cereals ready-to-eat, KELLOGG, KELLOGG'S PRODUCT 19	08058	20.00	Beef, ground, 75% lean meat/25% fat, patty, cooked, broiled	23578	2.81
Cereals ready-to-eat, KELLOGG, KELLOGG'S SPECIAL K	08067	19.52	Beef, ground, 85% lean meat/15% fat, patty, cooked, broiled	23568	2.64
Cereals ready-to-eat, GENERAL MILLS, TOTAL Corn Flakes	08246	20.00	Lamb, domestic, leg, whole (shank and sirloin), separable lean only, trimmed to 1/4″ fat, choice, cooked, roasted	17014	2.64
Mollusks, oyster, eastern, wild, raw	15167	19.46	Fish, roughy, orange, cooked, dry heat	15232	2.31
Cereals ready-to-eat, KELLOGG, KELLOGG'S ALL-BRAN Original	08001	18.80	Beef, chuck, blade roast, separable lean and fat, trimmed to 1/4″ fat, all grades, cooked, braised	13050	2.28
Snacks, CHEX mix	19033	12.42	Fish, tuna, light, canned in oil, drained solids	15119	2.20
Crustaceans, crab, Alaska king, cooked, moist heat	15137	11.51	Lamb, domestic, rib, separable lean only, trimmed to 1/4″ fat, choice, cooked, roasted	17034	2.16
Cereals ready-to-eat, GENERAL MILLS, TOTAL Raisin Bran	08247	10.89	Fish, swordfish, cooked, dry heat	15111	2.02
Cereals ready-to-eat, GENERAL MILLS, WHEATIES	08089	10.00	Fish, catfish, channel, cooked, breaded and fried	15011	1.89
Cereals ready-to-eat, GENERAL MILLS, FROSTED WHEATIES	08266	10.00	Fast foods, hamburger, regular, double patty, with condiments	21111	1.55
Chicken, broilers or fryers, giblets, cooked, simmered	05022	9.44	Fish, halibut, Atlantic and Pacific, cooked, dry heat	15037	1.37
Fish, sardine, Atlantic, canned in oil, drained solids with bone	15088	8.94	Soy milk, fluid	16120	1.22
Fish, salmon, sockeye, cooked, dry heat	15086	5.80	Fast foods, cheeseburger, regular, double patty and bun, plain	21094	1.20
Cereals ready-to-eat, KELLOGG, KELLOGG'S COCOA KRISPIES	08014	6.94	Fish, tuna salad	15128	1.20
Fish, trout, rainbow, farmed, cooked, dry heat	15241	4.96	Fast foods, cheeseburger, large, single patty, with condiments and vegetables	21098	1.17
Fish, Pollock, walleye, cooked, dry heat	15067	4.20	Cheese, cottage, lowfat, 2% milkfat	01015	0.71

[a] Data from USDA National Nutrient Database for Standard Reference, Release 19.

Table 11.2 Dietary Reference Intakes for Vitamin B_{12}

Life stage	DRI (μg d^{-1})
Infants (months)	
0–6	0.4
7–12	0.5
Children (years)	
1–3	**0.9**
4–8	**1.2**
Males (years)	
9–13	**1.8**
14–18	**2.4**
19–30	**2.4**
31–50	**2.4**
51–70	**2.4**
>70	**2.4**
Females (years)	
9–13	**1.8**
14–18	**2.4**
19–30	**2.4**
31–50	**2.4**
51–70	**2.4**
>70	**2.4**
Pregnancy (years)	
≤18	**2.6**
19–30	**2.6**
31–50	**2.6**
Lactation (years)	
≤18	**2.8**
19–30	**2.8**
31–50	**2.8**

Bold type: Recommended Dietary Allowance; ordinary type: adequate intake (AI).

Source: Food and Nutrition Board, Institute of Medicine, *Dietary Reference Intake for Thiamin, Ribiflavin, Naicin, Vitamin B_6, Folate, Vitamin B_{12}, Pantothenic Acid, Biotin, and Choline*, National Academy of Sciences Press, Washington, DC, 2000, Chap. 9.

Table 11.3 Vitamin B_{12}-Dependent Enzymes

Class I enzymes (Mutases)
Glutamate mutase (GluM, EC 5.4.99.1)
α-Methyl-eneglutarate mutase (MGM, EC 5.4.99.4)
Methyl-malonyl CoA mutase (MMCM, EC 5.4.99.2)
Isobutyryl CoA mutase (ICM, EC 5.4.99)

Class II enzyme (Eliminases)
Propanediol dehydratase (diol dehydratase, DD, EC 4.2.1.28)
Glycerol dehydratase (GD, EC 4.2.1.30)
Ethanolamine ammonia lyase (EAL, EC 4.3.1.7)
Ribonucleoside triphosphate reductase (RTPR, EC 1.17.4.2)

Class III enzymes (Aminomutases)
β-Lysine-5,6-aminomutase (LAM, EC 5.4.3.3)
D-ornithine-4-5-aminomutase (OAM, EC 5.4.3.4)

Source: Brown, K. L., Chemistry and enzymology of vitamin B-12, *Chem. Rev.*, 105, 2075, 2005.

are mutases that catalyze carbon skeleton rearrangements and the migrating group is a carbon fragment. Cleavage of a C–C bond occurs and the receiving carbon has two hydrogens.[13] Most Class II enzymes are eliminases that catalyze the migration and elimination of a hydroxyl or amino group with the cleavage of a C–O on C–N bond.[13] The receiving carbon has a hydroxyl substituent. Class III enzymes are aminomutases that catalyze amino group migration to an adjacent carbon that bears two hydrogens with the cleavage of a C–N bond. The amino mutases also require pyridoxal phosphate (PLP).[13]

AdoCbl-dependent reactions include conversion of methylmalonyl CoA to succinyl CoA by methylmalonyl CoA mutase in the degradation of propionate and the conversion of leucine to 3-aminoisocapronate by leucine mutase. MeCbl functions with methionine synthetase in the methylation of homocysteine in methionine synthesis. Vitamin B_{12} is required for the enzymatic removal of the methyl group from methylfolate, which regenerates tetrahydrofolate required for formation of 5,10-methylene tetrahydrofolate. 5,10-Methylene tetrahydrofolate is the source of thymidylate (dTMP) synthesis. Therefore, vitamin B_{12} deficiency causes folate to be trapped as methylfolate. Adequate amounts of 5,10-methylene tetrahydrofolate become unavailable for DNA synthesis. The folate-trap mechanism and its biochemistry are explained in detail by Hebert.[3] In both folate and vitamin B_{12} deficiency, megaloblastic changes in red blood cells are similar because of defective synthesis of DNA.

11.2 Properties

11.2.1 Chemistry

11.2.1.1 General properties

Vitamin B_{12} is the collective name for cobalt-containing corrinoids with the biological activity of CNCbl. The corrin structure includes four reduced pyrrole rings joined by three methene bridges with two pyrroles linked directly. The central cobalt atom is bound by coordinate linkages to the nitrogen atoms of the four pyrrole rings. The corrin ring is structurally similar to heme except it has one less α-methene bridge and has cobalt in place of iron.[3] The structural formula of cobalamin is shown in Figure 11.1. Cyanocobalamin (CNCbl) is the permissive name for vitamin B_{12}. Nutritionally, vitamin B_{12} includes all cobalamins biologically active in the human. In CNCbl, the β-position of the cobalt atom is occupied by a cyano-ligand (CN^-). In addition to CN^-, the β-position may be occupied by OH^- to form hydroxocobalamin (OHCbl), water to form aquocobalamin (H_2OCbl), NO_2 to form nitrocobalamin (NO_2Cbl), deoxyadenosyl to form coenzyme B_{12} (AdoCbl), methyl to form methylcobalamin (MeCbl), and SO_3 to form sulfitocobalamin (SO_3Cbl). Use of the terms vitamin B_{12a} (OHCbl), vitamin B_{12b} (H_2OCbl), and vitamin B_{12c} (NO_2Cbl) are not recommended by International Union of Pure and Applied Chemistry—International Union of Biochemistry (IUPAC-IUB).[14–16]

General properties of the various cobalamins important in metabolism are given in Table 11.4. CNCbl is a tasteless, odorless, red crystalline substance with good water solubility (1 g 80 mL^{-1} at 25°C). In the amorphous form, the vitamin is hygroscopic and absorbs about 12% by weight of water.[19] The vitamin is soluble in alcohols, phenols, and other polar solvents with hydroxy groups. CNCbl is not soluble in other organic solvents including acetone, ether, and benzene. Crystals do not melt, but decompose above 200°C. The USP standard is CNCbl.

CNCbl and OHCbl are the forms of vitamin B_{12} available for medical use and fortification by the food industry. CNCbl is predominantly used in vitamin preparations, supplements, medical foods, and fortified foods because of its better stability compared to OHCbl. OHCbl is used to treat specific disease states including tobacco amblyopia and optic neuropathy.[3,20]

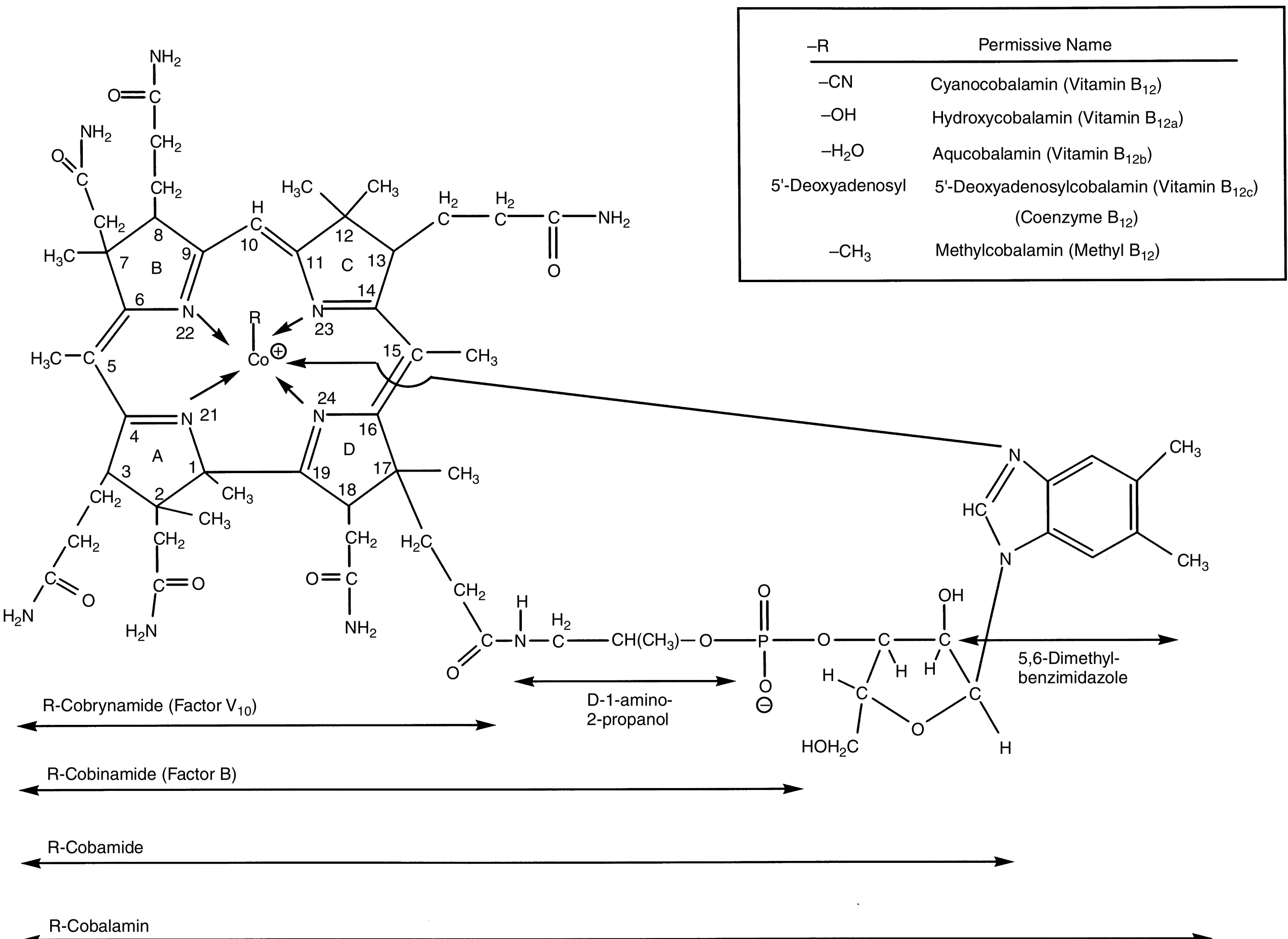

Figure 11.1 Structure of vitamin B_{12}.

Table 11.4 Physical Properties of Vitamin B12

Substance[a]	Molar mass	Formula	Solubility	Crystal form	Absorbance[b]			Solvent
					λ_{max} (nm)	$E^{1\%}_{1\,cm}$	ε x 10^{-3}	
Cyanocobalamin B_{12}	1355.38	$C_{63}H_{88}CoN_{14}O_{14}P$	Soluble in water	Dark red hygroscopic	278	115	[15.6]	Water
CAS No. 68-19-9			(1 g 80 mL^{-1})	Darkens at	361	204	[27.6]	Water
10152				210–220° C	551	64	[8.7]	Water
Hydroxocobalamin B_{12a}	1346.37	$C_{62}H_{89}CoN_{13}O_{15}P$	Moderately soluble in	Dark red	279	[141]	19.0	Water
CAS No. 13422-51-0			water	orthorhombic	325	[85]	11.4	Water
4854			Insoluble in acetone,	Darkens at 200°C	359	[153]	20.6	Water
			ether, petroleum, ether,		516	[66]	8.9	Water
			benzene		537	[71]	9.5	Water
Aquacobalamin B_{12b}	1347.0	$C_{62}H_{90}CoN_{13}O_{15}POH$			274	[153]	20.6	Water
CAS No. 13422-52-1					317	[45]	6.1	Water
					351	[197]	26.5	Water
					499	[60]	8.1	Water
					527	[63]	8.5	Water
Nitrocobalamin B_{12c}	1374.6	$C_{62}H_{88}CoN_{14}O_{16}P$		Red crystalline solids	352	153	[21.0]	Water
					528	60	[8.4]	Water
					357	139	[19.1]	N NaOH
					535	63	[8.7]	0.01 N NaOH
Sulfitocobalamin	1409.5	$C_{62}H_{89}CoN_{13}O_{17}PS$			275	328	[46.2]	Water
CAS No. 15671-27-9					365	130	[18.3]	Water
4854					418	49	[6.9]	Water
					516	61	[8.6]	Water
Adenosylcobalamin	1579.6	$C_{72}H_{100}CoN_{18}O_{17}P$	Soluble in ethanol, phenol	Yellow-orange	288	[115]	18.1	Water, pH 7.0
Cobamamide			Insoluble in acetone, ether	6-faced crystal	340	[78]	12.3	Water, pH 7.0
CAS No. 13870-90-1			dioxane		375	[60]	10.9	Water, pH 7.0
2513					522	[51]	8.0	Water, pH 7.0
Methylcobalamin	1344.4	$C_{63}H_{91}CoN_{13}O_{14}P$		Bright red	266	[148]	19.9	Water, pH 7.0
CAS No. 13422-55-4					342	[107]	14.4	Water, pH 7.0
6125					522	[70]	9.4	Water, pH 7.0
					264	[184]	24.7	0.1 N HCl
					304	[170]	22.9	0.1 N HCl
					462	[71]	9.6	0.1 N HCl

[a] Common or generic name; CAS No.: Chemical Abstract Service number, bold print designates the Merck Index monograph number.
[b] Values in brackets are calculated from corresponding $E^{1\%}_{1\,cm}$ values.

Sources: Budavari, S., *The Merck Index*, 13th ed., Merck and Company, Whitehouse Station, New Jersey, 2001, p. 1785; Friedrich, W., Vitamin B-12, In *Vitamins*, Walter de Gruyter, Berlin, 1998, chap. 13; Committee on Food Chemicals Codex, *Food Chemicals Codex*, 5th ed., National Academy of Sciences, Washington, DC, 2004, p. 496.

11.2.1.2 Spectral properties

Aqueous solutions of CNCbl have absorption maxima at 278, 361, and 551 nm.[18,21,22] The spectra of CNCbl, H_2OCbl, and OHCbl are similar. Maximal absorbance is in the region of 350–368 nm.[18] AdoCbl and MeCbl show less intense absorption around 360 nm and change color from red to yellow in weakly acidic solutions.[18] Spectroscopic properties including nuclear magnetic resonance (NMR) spectra, magnetic circular dichroism, and Raman spectroscopy were reviewed by Brown.[13] X-ray cyrstallography studies relating to structural characterization were detailed in a review paper by Perry and Marques.[23]

11.2.2 Stability

Crystalline forms of vitamin B_{12} are stable when protected from light.[18,24] CNCbl is the most stable form of vitamin B_{12}.[25] Light exposure cleaves the cyanide with the production of OHCbl.[26] Other ions or groups can attach to the cobalt with production of other cobalamins. In excess cyanide, it can replace other moieties bound to the β-position of the cobalt. CNCbl has optimum stability at pH 4.0–4.5.[27] It is stable to autoclaving between pH 4.0 and 7.0.[26] Severe alkaline and acid conditions, ultraviolet (UV), or strong visible light, and oxidizing agents inactivate the vitamin.[27] There is some indication in the literature that stability can be decreased in multinutrient preparations in the presence of thiamin, niacin, and ascorbic acid. However, the literature is not definitive.[20,25] However, in the solid state, CNCbl as well as OHCbl and cobinamide are quite stable.[28]

Stability of vitamin B_{12} in multivitamin tablets is dependent upon the type and amount of coating and increases expiration time values. Coated tablets provided expiration times for vitamin B_{12} of up to 1000 days.[29] In serum, addition of potassium cyanide (KCN) can restore and/or protect vitamin B_{12} activity.[30] Komaromy-Hiller et al.[31] indicated that vitamin B_{12} in serum is highly unstable. These authors recommended that serum samples be frozen and protected from light to ensure maximum stability (Figure 11.2).

Vitamin B_{12} is stable to most food processing operations, but, like all water-soluble vitamins, it can be lost through leaching during boiling or in purge from meats during cooking. Early studies by Helendoorn et al.[32] showed that vitamin B_{12} was stable to retorting in meals containing beef. Others have reported good stability in beef cooked by home cooking procedures.[33] Effects of microwave cooking on the stability of vitamin B_{12} was thoroughly investigated by Watanabe et al.[34,35] From 30% to 40% of vitamin B_{12} was degraded in beef, pork,

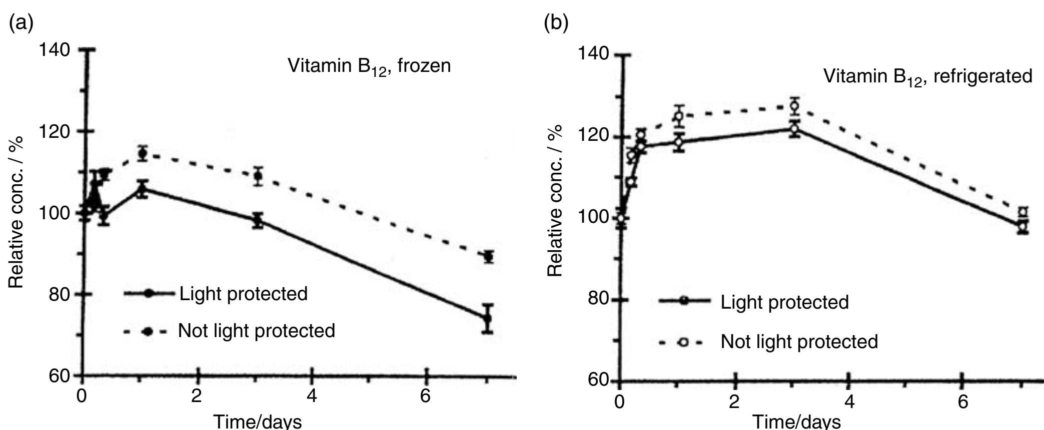

Figure 11.2 Stability of serum vitamin B_{12} under different storage conditions. (Reproduced from Komaromy-Hiller, G., Nuttall, K. L., and Ashwood, E. R., *Ann. Clin. Lab. Sci.*, 27, 249, 1997. With permission.)

and milk by microwave processing. Two degradation products were associated with microwave heating of OHCbl.[34] Characterization of the major degradation product indicated slight biological activity (12%–13% of OHCbl) and was not toxic or antagonistic in mammals.[35]

11.2.3 Bioavailability

Bioavailability of vitamin B_{12} can be decreased by vitamin B_{12}-binding proteins present in certain foods that have some resistance to digestive processes, thus decreasing uptake by the intrinsic factor. Specific membrane receptors in the ileum will not attach to vitamin B_{12} proteins because of high specificity for the intrinsic factor complex.[36] In the elderly, decreased secretion of gastric enzymes can increase the effects of food-origin vitamin B_{12} binders.[37]

While most foods have low or vitamin B_{12}-binding capacity, raw egg yolk and white have specific vitamin B_{12}-binding proteins.[38] These binders were shown to be inactivated by heating. Other foods studied that did not possess apparent vitamin B_{12}-binding capacity included mutton,[39] chicken,[40] fish,[41] milk,[42] and fortified bread.[42]

11.3 Methods

Available methods for assay of vitamin B_{12} include polarographic, spectrophotometric, electrophoretic, various chromatographic procedures including paper, thin-layer, open-column, gas chromatography (GC), liquid chromatography (LC), and LC combined with mass spectrometry (LC-MS) procedures, microbiologic, and radio-ligand binding procedures. Almost all available data of vitamin B_{12} in food has been obtained by microbiological assay.[9] Assay of naturally occurring vitamin B_{12} in most biological matrices is difficult owing to the low concentrations normally present. Except for organ meats, vitamin B_{12} levels usually are less than 10 μg 100 g^{-1} and often below 1.0 μg 100 g^{-1}. Relatively poor sensitivity by most detector modes combined with low, natural levels in animal products makes vitamin B_{12} assay by LC difficult even if preconcentration, usually by immunoaffinity columns, is used. For clinical tissue and serum samples, radio-ligand binding assays with ^{57}Co-labeled CNCbl are routine and dependable. However, radioassay kits for clinical assay are not reliable for assay of food (Section 11.3.2). Normal vitamin B_{12} content of serum ranges from 150 to 750 pg μL^{-1}.[1]

Liquid chromatography can be conveniently applied to the assay of vitamin B_{12} in high concentration supplements and pharmaceutical preparations.[43–53] LC can effectively resolve natural cobalamins. However, owing to low levels of the analytes, isotope dilution and radio-ligand binding assays were often used in early LC studies to quantitate eluted cobalamins. At this time, only limited use has been made of mass spectrometry in conjunction with LC. However, Lindemans[26] points out that soft ionization techniques useful for nonvolatile and thermolabile compounds will be applicable to LC-MS methods for vitamin B_{12}. Excellent discussions of LC methods useful for vitamin B_{12} assay were presented by Lindemans.[26]

Regulatory and handbook procedures are summarized in Table 11.5. These methods use spectrophotometric, microbiological, and LC approaches for the assay of CNCbl in pharmaceuticals and microbiological assay for foods.[54–60]

11.3.1 AOAC International methods (Lactobacillus delbrueckii)

AOAC International[56] methods use microbiological assay with *Lactobacillus delbrueckii* (*leichmannii*) ATCC 7830. The methodology was originally collaborated for use on vitamin preparations, but the Association of Official Analytical Chemists (AOAC) Task Force on Methods for Nutrition Labeling[61] recommended the procedure for use on all food matrices.

Table 11.5 Regulatory and Handbook Methods of Analysis for Vitamin B_{12}

Source	Form	Methods and application	Approach	Most current cross-reference
***U.S. Pharmacopeia, National Formulary*, 2006, USP 29/NF 24 Dietary Supplements Official Monographs**[54]				
1. Pages 2392, 2394–2395, 2398, 2403, 2415–2416	Cyanocobalamin	Cyanocobalamin in oil- and water-soluble vitamin capsules/tablets, oral solution w/wo mineral	Method 1–HPLC 550 nm Method 2–Microbiological	None
2. Pages 2427, 2430–2431, 2434, 2436	Cyanocobalamin	Cyanocobalamin in water-soluble capsules/tablets, oral solution w/wo mineral	Method 1–HPLC 550 nm Method 2–Microbiological	None
3. Pages 576–577, 2548–2549	Cyanocobalamin	Cyanocobalamin Co^{57} capsule/oral solution	Microbiological	None
4. Page 604	Cyanocobalamin	Cyanocobalamin injection	Spectrophotometric 361 nm	None
5. Pages 603–604	Cyanocobalamin	Cyanocobalamin (NLT 96.0%, NMT 100.5%)	Spectrophotometric 361 nm	None
British Pharmacopoeia, 2007[55]				
1. Pages 608–609	Cyanocobalamin	Cyanocobalamin	LC 361 nm	None
2. Page 2485	Cyanocobalamin	Cyanocobalamin coated tablets	LC 361 nm, 550 nm	None
3. Pages 3177–3178, 3179-3180	Cyanocobalamin	Cyanocobalamin [^{57}Co] capsules and solution	LC 361 nm and Gamma detector for ^{57}Co	None
4. Pages 3178–3179, 3180	Cyanocobalamin	Cyanocobalamin [^{58}Co] capsules and solution	LC 361 nm and Gamma detector for ^{58}Co	None
***AOAC Official Methods of Analysis*, 18th ed., 2005**[56]				
1. 45.2.02	Cyanocobalamin	AOAC Official Method 952.20, Cobalamin (Vitamin B_{12} Activity) in Vitamin Preparations	Microbiological	*J. Assoc. Off. Anal. Chem.*, 42, 529, 1959[57]
2. 50.1.20	Cyanocobalamin	AOAC Official Method 986.23, Cobalamin (Vitamin B_{12} Activity) in Milk-Based Infant Formula	Microbiological	*J. Assoc. Off. Anal. Chem.*, 69, 777, 1986[58]
American Association of Cereal Chemists, Approved Methods, 10th ed., vol. 2, 2000[59]				
1. AACC 86–40	Cyanocobalamin in cereal products	Vitamin B_{12}, Microbiological Method	Microbiological	None
***Food Chemical Codex*, 5th ed., 2004**[60]				
1. Pages 496–497	Cyanocobalamin	NLT 96.0% NMT 100.5%	Spectrophotometric 361 nm	None

AOAC Official Method 952.20, Cobalamin (Vitamin B_{12} Activity) in Vitamin Preparations, Microbiological Method, Official Methods of Analysis of AOAC International, 45.2.02.

L. delbrueckii growth response is sensitive enough to quantitate CNCbl at concentrations approaching 1.0 pg mL^{-1} of assay growth media. This sensitivity is sufficient to quantitate vitamin B_{12} in foods containing less than 0.5 µg 100 g^{-1}.[9]

Vitamin B_{12} active compounds are extracted in phosphate buffer containing 1.3 g Na_2HPO_4, 1.2 g citric acid, and 1.0 g anhydrous sodium metabisulfite per 100 mL. Extracting solutions must contain a reducing agent such as metabisulfite or ascorbic acid to protect the cobalamins throughout the extraction.[62,63] Many methods incorporate sodium cyanide to ensure the conversion of the more labile OHCbl, the most predominant form in foods, to the more stable dicyanocobalamin $(CN)_2Cbl$.[27] Method 952.20 does not use sodium cyanide. Of importance, sodium metabisulfite must be at a concentration less than 0.03 mg mL^{-1} in the final assay solution to avoid inhibition of *L. delbrueckii* growth. The extraction is completed by homogenizing the sample in the extraction solution, autoclaving the mixture at 121°C for 10 min, adjusting the cooled mixture to pH 4.5, diluting with water to a vitamin B_{12} concentration of approximately 0.2 ng mL^{-1}, and filtering. Standard microbiological assay techniques are used to quantitate the vitamin (AOAC Method 960.46).

AOAC Official Method 986.23 (50.1.20) "Cobalamin (Vitamin B_{12} Activity) in Milk-Based Infant Formula" is basically the same as Method 952.20. At this point in time, AOAC International has not collaborated the method for use on other types of infant formula; however, the AOAC Task Force on Methods for Nutrient Labeling listed Method 986.23 as acceptable for all infant formulas.[61]

L. delbrueckii has variable response to various cobalamins. Muhammad et al.[62] determined the growth response of *L. delbrueckii* to CNCbl, OHCbl, SO_3Cbl, $(CN)_2Cbl$, NO_2Cbl, MeCbl, and AdoCbl since the potential exists for these cobalamins to be present in extracts of biological samples prepared with the addition of sodium cyanide, potassium cyanide, sodium metabisulfite, or sodium nitrite. Similar growth response was found for CNCbl, OHCbl, SO_3Cbl, $(CN)_2Cbl$, and NO_2Cbl. However, AdoCbl produced a greater response and MeCbl a lesser growth response (Figure 11.3). When CNCbl is used as the calibration standard in the *L. delbrueckii* assay, accurate determinations can be obtained for OHCbl, SO_3Cbl, CNCbl, and AdoCbl when the extracting solution contains excess cyanide; however, MeCbl will be underestimated. In the presence of sodium metabisulfite or sodium nitrite, only OHCbl, SO_3Cbl, and CNCbl can be assayed with confidence. AdoCbl will be overestimated if extracted in the presence of sodium metabisulfite or sodium nitrite because these compounds do not modify AdoCbl. Conversely, MeCbl, which is also stable in the presence of metabisulfite and nitrite, will be underestimated. Excess cyanide converts OHCbl, SO_3Cbl, and AdoCbl to the $(CN)_2Cbl$ form, and MeCbl remains unchanged.[62] Because AdoCbl and OHCbl are the predominant cobalamins in food,[62,64] the AOAC International assay potentially can overestimate naturally occurring vitamin B_{12} activity since cyanide is omitted from the extracting solution.

Muhammad et al.[62] concluded that vitamin B_{12} activity cannot be accurately measured by *L. delbrueckii* using CNCbl as the calibration standard if AdoCbl and/or MeCbl are present in the sample extract. To circumvent the inability of commonly used extractants to convert AdoCbl and MeCbl to cobalamins with equal response to CNCbl in the *L. delbrueckii* assay, photoconversion of these forms to OHCbl was suggested before assay. Complete conversion occurs under exposure to fluorescent light within 1 h.[62] This approach has not been incorporated into published work or analysis of vitamin B_{12} activity in food. However, improvement in the reliability of data obtained by assay with *L. delbrueckii* might be possible through addition of this simple extraction step.

L. delbrueckii can utilize vitamin B_{12} analogs, deoxyribonucleotides, and deoxyribonucleosides in addition to biologically active coablamine. Older literature suggests that dilution

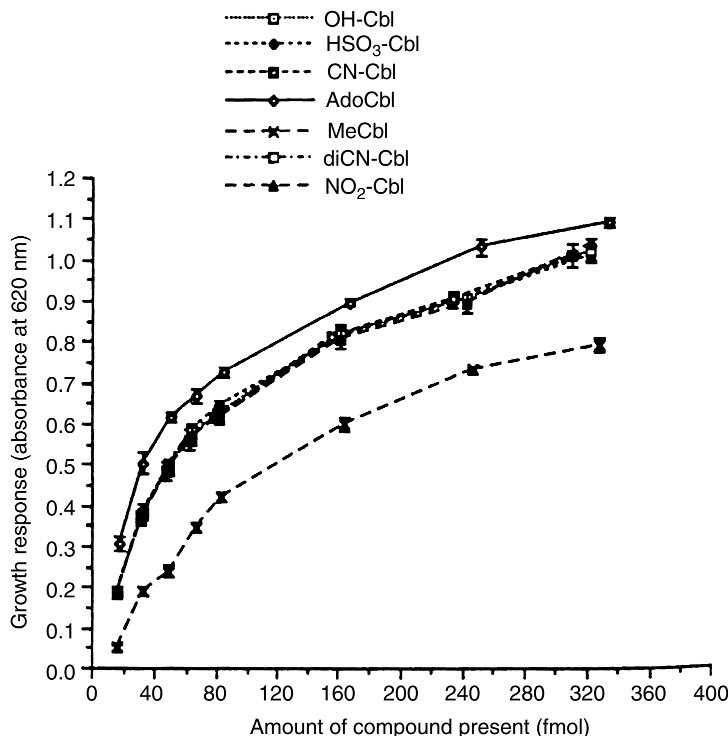

Figure 11.3 Growth responses of *Lactobacillus delbrueckii* (ATCC 7830) to OHCbl, HSO_3Cbl, CNCbl, AdoCbl, MeCbl, DiCNCbl, and NO_2Cbl. (Reproduced from Muhammad, K., Briggs, D., and Jones, G., *Food Chem.*, 48, 427, 1993. With permission.)

of dioxyriboside levels (e.g., thymidine) to less than 1 μg mL^{-1} of the assay medium will eliminate the effect.[65] Comparative studies on animal products assayed by *L. delbruekii* and *Ochromonas malhamensis*, a protozoan considered to be the most specific assay organism for measurement of vitamin B_{12} activity, indicated little difference in the values obtained by the two organisms.[66] The ease of using *L. delbrueckii*, compared to the protozoan, makes it a clear choice for vitamin B_{12} analysis in most laboratories.

11.3.2 Radio-ligand binding assays

Radio-ligand binding assays have been routinely used for blood and tissue analysis since early work by Rothenberg,[67] Barakat and Elkins,[68] and Lau et al.[69] established the use of intrinsic factor as the binding protein and [^{57}Co]CNCbl as the radiolabeled ligand for a competitive binding assay for vitamin B_{12} in serum. Other binding proteins in addition to intrinsic factor have been used, including transcobalamin I (TC-I), transcobalamin II (TC-II), and haptocorrin or R-binder from saliva. Early studies established that such binding proteins were not specific for Cbl and bound nonbiologically active cobalamins. Porcine intrinsic factor is routinely used as the binding protein in most clinical applications.

Specific radioimmunoassays (RIA) are available for CNCbl[70] and AdoCbl.[71] The RIA methods are based on monospecific antisera and eliminate cross-reaction with other cobalamins. Such assays are not in routine use in most clinical laboratories.

Studies by several research groups indicated that radio-ligand binding assays with intrinsic factor could be used for food analysis.[63,72–79] The early research established extraction procedures necessary to liberate bound cobalamins from food matrices. When

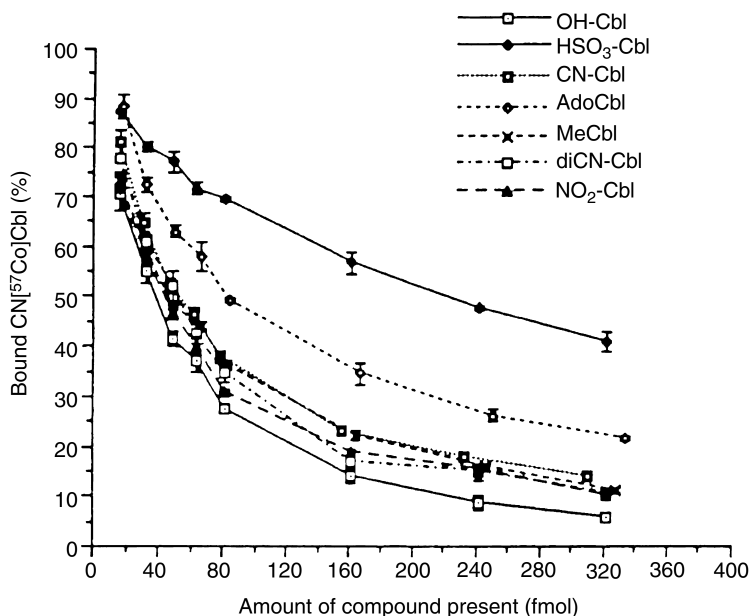

Figure 11.4 The relative binding affinity of OHCbl, HSO_3Cbl, CNCbl, AdoCbl, McCbl, DiCNCbl, and NO_2Cbl with porcine intrinsic factor. (Reproduced from Muhammad, K., Briggs, D., and Jones, G., *Food Chem.*, 48, 423, 1993. With permission.)

optimized extraction procedures were used, radio-ligand and microbiological procedures showed agreement for most foods; however, differences were noted often enough to conclude that the two methods are not universally interchangeable for the assay of all foods. Differences in results between the two methods indicated that radio-ligand assay gave lower values than microbiological assay in situations when variation existed.[80] This trend was postulated to be due to inefficient extraction of vitamin B_{12} in chemical forms bindable to the intrinsic factor and to the lower specificity of *L. delbrueckii*.[80]

Muhammad et al.[81] studied the relative binding affinities of CNCbl, CN_2Cbl, OHCbl, SO_3Cbl, NO_2Cbl, AdoCbl, and MeCbl with porcine intrinsic factor. Equivalent binding was noted for CNCbl, CN_2Cbl, NO_2Cbl, and MeCbl ($p > 0.2$) (Figure 11.4). Significantly different binding affinities were found for OHCbl, SO_3Cbl, and AdoCbl. Therefore, porcine intrinsic factor cannot be used as the binding protein with CNCbl as the calibration standard unless equal cyanide is used in the extraction procedure to convert OHCbl, SO_3Cbl, and AdoCbl to CN_2Cbl.[81] Effects of excess potassium cyanide, sodium metabisulfite, and sodium nitrite on the conversion of cobalamins are given in Table 11.6.

A protein-binding assay developed by Alcock et al.[82] appears to be useful for fortified foods in which CNCbl is the predominant cobalamin. The procedure uses a R-protein-enzyme conjugate and microtitration plate techniques for quantitation of CNCbl. Since R-proteins bind Cbl and Cbl analogs, the assay has not been applied to nonfortified foods. Applications to fortified breakfast cereals gave a limit of detection of 9 pg per well and a quantitation limit of 0.09 μg 100 g^{-1}.

Currently, protein-ligand binding assays are not routinely used for food analysis. Although the Alcock et al.[82] procedure could be a time- and labor-saving method for analysis of vitamin B_{12} in fortified foods, the lack of a commercial source of the R-protein enzyme conjugate is a hindrance to use of the assay by most laboratories involved with analysis of vitamin B_{12} in foods. The microbiological assay by *L. delbrueckii* continues to be widely used for food analysis.

Table 11.6 Conversion[a] of Cobalamins by Excess Potassium Cyanide, Sodium Metabisulfite, and Sodium Nitrite

Before conversion	+KCN	After conversion $+Na_2S_2O_5$	$+NaNO_2$
OHCbl	diCNCbl	HSO_3Cbl	NO_2Cbl
HSO_3Cbl	diCNCbl	HSO_3Cbl	HSO_3Cbl
CNCbl	diCNCbl	HSO_3Cbl	CNCbl
AdoCbl	diCNCbl	AdoCbl	AdoCbl
MeCbl	MeCbl	MeCbl	MeCbl

[a] Based on absorption spectrophotometry of aqueous solutions. Spectra were determined 30 min after addition of the compound and incubation in the dark. The ratio, in terms of number of moles of KCN, $Na_2S_2O_5$ and $NaNO_2$ to each cobalamin was 50:1, 1.5:1, respectively. Reproduced from Muhammad, K., Briggs, D., and Jones, G., The appropriateness of using cyanocobalamin as calibration standards in competitive binding assays of vitamin B_{12}, *Food Chem.*, 48, 423, 1993. With permission.

11.3.3 *Advances in the analysis of vitamin B_{12}*

11.3.3.1 *Spectroscopic and chemiluminescence methods*

Spectrophotometric procedures are available for the assay of high concentration pharmaceutical products. Özgür and Koyuncu[83] used second derivative spectrophotometry to assay vitamin B_6, thiamin, and vitamin B_{12} in pharmaceuticals. Calibration graphs were linear up to 20 μg mL^{-1} for thiamin at 228.9 nm, vitamin B_6 at 309.6 nm, and vitamin B_{12} at 361.7 nm. Analysis of tablets containing 1 mg of vitamin B_{12} gave results close to the label amount with a %RSD of 3.58. Nepote et al.[84] developed a simultaneous analysis of vitamin B_6, dexamethasone, and vitamin B_{12} on the basis of UV-vis spectral measurements combined with partial least squares (PLS-1) multivariate calibration. Application to injectables containing vitamin B_{12} gave results comparable to results obtained by capillary electrophoresis. Liu et al.[85] presented a quantitative method for vitamin B_{12} on the basis of the energy transfer fluorescence quenching of Acridino orange-Rhodamine B. Diminished fluorescence intensity of the Rhodamine B occurred in the presence of vitamin B_{12}. Assay of injectables was successful with %RSD of 1.39–1.93 and high recoveries.

Costs and specificity problems associated with the radio-ligand assay for food analysis led to the development of assays based on fluorescent derivatives of vitamin B_{12}. Watanabe et al.[86] used 6,7-dimethoxy-1-methyl-2(1H)-quinoxaline-3-propionylcarboxylic acid hydrazide (DMEQ) to produce a highly fluorescent vitamin B_{12} derivative that proved useful for analysis of vitamin B_{12}. Binding of the derivative with hog intrinsic factor yielded a fluorescence dilution assay based on competitive binding of vitamin B_{12} and the vitamin B_{12}-fluorescent derivative for sites on the intrinsic factor. Assay results favorably compared with those obtained by chemiluminescence and microbiologically. Li and Chen[87] were able to assay pharmaceutical preparations of vitamin B_{12} by measuring the relatively weak fluorescence of cyanocobalamin at Ex $\lambda = 275$, Em $\lambda = 305$.

Several methods have been published that use chemiluminscence for the assay of vitamin B_{12}.[88–93] Most are applicable to flow injection analysis (FIA) and, therefore, are rapid throughput procedures. As an example, Song and Hou[93] provided a method based on the enhancement of cobalt (II) on the chemiluminescence reaction between luminol and dissolved oxygen in the flow injection system. The method was very sensitive with a detection limit of 5×10^{-11} g L^{-1}. The method was applicable to pharmaceuticals, serum, and foods. The authors' evaluation indicated the speed and simplicity of the procedure, making it useful as a routine analytical method. Watanabe et al.,[90] in earlier research, provided an assay based on a commercially available chemiluminescence vitamin B_{12} analyzer. This system (ACS 180 PLUS, Chiron Diagnostics) relies on vitamin B_{12} labeled with a chemiluminescent acridium ester. Full details of the method were not provided.

11.3.3.2 Capillary electrophoresis

The first reported use of capillary electrophoresis (CE) for assay of vitamin B_{12} compared high performance capillary electrophoresis (HPCE) to LC to the quantitation of CNCbl, AdoCbl, MeCbl, OHCbl, and cyanocobinamide.[94] The detection limit of the HPCE procedure was 20 μg mL^{-1} versus 100 pg mL^{-1} for LC combined with radioisotope dilution. Resolution and within-run reproducibility was quite good for both systems. Because of the differences in detection limits, the authors clearly showed that LC coupled with radioisotope dilution would be preferred for low concentration biological samples. However, HPCE was the choice for high concentration pharmaceuticals because of speed and simplicity. Few applications of CE for vitamin B_{12} assay have been published. However, Baker and Miller-Ihli[95] showed the potential of capillary zone electrophoresis coupled with inductively coupled plasma mass spectrometry (CZE-ICP-MS) for determining CNCbl in vitamin preparations. The CZE-ICP-MS system resolved AdoCbl, cobinamide, OHCbl, MeCbl, and CNCbl (Figure 11.5). Micellar electrokinetic chromatography (MEKC) with ICP-MS provided a rapid screening method for supplements and rapidly resolved CNCbl from free cobalt (Figure 11.6).[95]

11.3.3.3 High performance liquid chromatography and high performance liquid chromatography-mass spectrometry

Although radio-ligand binding and microbiological assay with *L. delbrueckii* are most commonly used for analysis of vitamin B_{12} in serum and tissues, excellent LC procedures are now available. Development of LC methodology has been hampered by the low sensitivity and selectivity of most detection modes for vitamin B_{12} active cobalamins. Therefore, most procedures incorporate cleanup and concentration steps to increase ability to assay low concentration natural products. Such extraction steps are often used for higher concentration pharmaceuticals. In spite of such problems related to instrumental analysis of vitamin B_{12}, methods with excellent figures of merit are available.

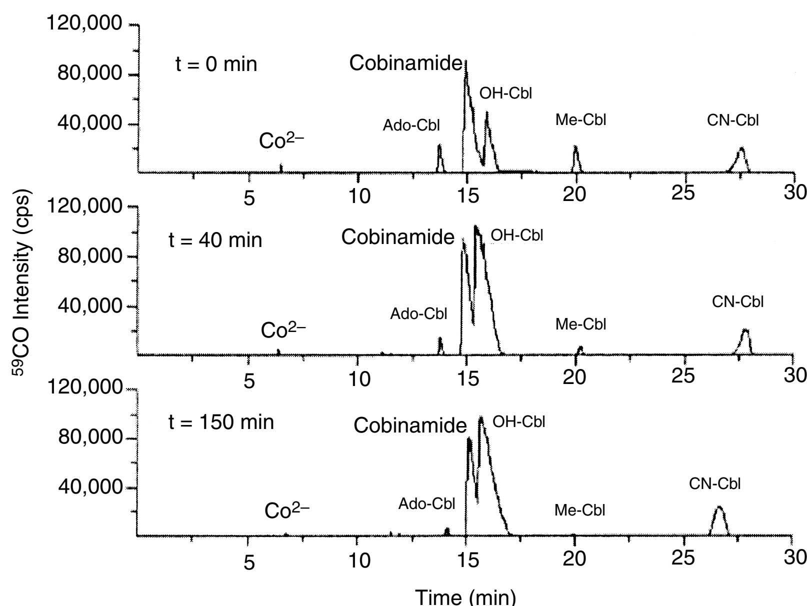

Figure 11.5 Electropherograms of a 200 μg mL^{-1} mixture of cobalamins and cobinamide dicyanide exposed to room temperature (20°C) and light (UV fluorescent lighting) from 0 to 150 min. (20 mM phosphate buffer, pH 2.5; run potential of 30 kV). (Reproduced from Baker, S. A. and Miller-Ihli, N. J., *Spectrochim. Acta Part B*, 55, 1823, 2000. With permission.)

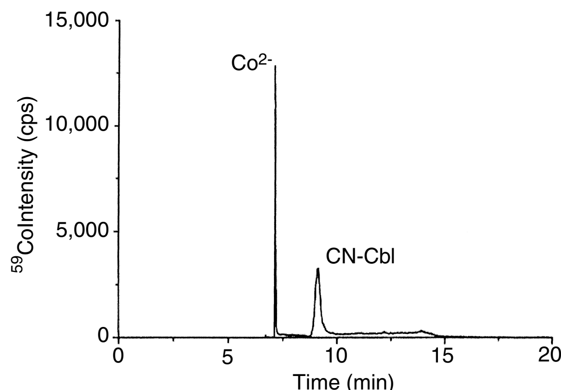

Figure 11.6 Electropherogram of an injectable vitamin supplement containing cyanocobalamin and free Co^{2+} using MEKC. (50 mM Tris + 12 mM SDS, pH 8.0; run potential of 30 kV). (Reproduced from Baker, S. A. and Miller-Ihli, N. J., *Spectrochim. Acta Part B*, 55, 1823, 2000. With permission.)

Relatively few LC-MS methods have been developed that capitalize on the greater selectivity and sensitivity provided by MS. More routine use of LC-MS for vitamin B_{12} assay undoubtedly will be forthcoming. Liquid chromatographic and LC-MS methods are summarized in Table 11.7.[47–51,96–110]

11.3.3.3.1 Extraction procedures for analysis of vitamin B12 by LC. Almost all extractions of vitamin B_{12} in natural products before assay by LC must incorporate a cleanup and/or concentration step to improve selectivity and sensitivity. Solid phase extraction,[47,96,98,107,109] ion-exchange[47,108] and immunoaffinity chromatography[101,102] have been successfully used. Pharmaceuticals can be solubilized into buffer and often directly injected without further treatment. For foods, various buffers,[96,98] trichloroacetic acid (TCA) solutions,[97] and enzyme extractions with α-amylase and pepsin[100,101,103] have been recommended. Investigators using enzyme digestion show that better release of protein-bound cobalamins is obtained compared to more traditional procedures. However, the enzyme digestion has not been routinely incorporated into vitamin B_{12} analysis protocols. The relative ease and simplicity of such enzyme digestions make their use an easy addition. The procedure used by Heudi et al.[101] included a 3 h digestion at 37°C with α-amylase and pepsin.

11.3.3.3.2 Chromatography parameters

Supports and Mobile Phases

Reversed-phase chromatography on C_{18} with a variety of buffers have been the basis of practically all LC procedures for vitamin B_{12} assay. However, Viñas et al.[110] studied an amide stationary phase (Discovery RP-Amide) for the speciation and determination of several cobalamins (CNCbl, AdoCbl, OHCbl, MeCbl) (Figure 11.7). The support was endcapped with trimethylsilyl, allowing residual silanol groups to react more strongly with cobalamins. Detection limits ranged from 0.015 to 0.03 µg mL^{-1} by photodiode array detection at 265 nm for AdoCbl and MeCbl, 351 nm for OHCbl, 361 nm for CNCbl and 550 nm to prevent matrix interferences.

Detection

Ultraviolet absorbance at 361 nm or absorbance in the visible range at 550 nm are frequently used for detection of cobalamins; however, lack of sensitivity and specificity of spectroscopic detection and weak fluorescence of cobalamins has hindered natural product LC applications. High sensitivity and quite good specificity of the *L. delbruekii* assay lessened

Table 11.7 LC and LC-MS Methods for the Analysis of Vitamin B_{12} in Foods, Pharmaceuticals, and Biologicals

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Foods					
Elemental diet/ cyanocobalamin	Add 60 mL deioinized water to 20 g sample, water-bath, 50 °C, add 10 g NaCl. Stand, 30 min. Dilute to 100 mL w/deionized water. Remove lipid w/ hexane. Clean up—Sep-Pak C_{18}	Capcellpak C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic H_2O:MeCN (87:13) Flow rate—0.6 mL min^{-1}	550 nm	%Recovery—95–99	*J. Chromatogr.*, 590, 359, 1992[96]
Liver, meat/ cyanocobalamin	Mix 5 g sample w/10 mL 3% TCA, homogenize and centrifuge. Recover aqueous phase, reextract residue. Combine the aqueous phases, dilute to 25 mL w/3% TCA, filter	Spherisorb C_{18} ODS-2, 5 µm, 15 cm × 4.6 mm Mobile phase—gradient Phosphate buffer (0.05 M, pH 4.2)–MeOH Flow rate—1.5 mL min^{-1}	Atomic absorption spectrometer 240.7 nm	DL—0.32 µg mL^{-1} %RSD—3.5	*Chromatographia*, 42, 566, 1996[97]
Foods/ cyanocobalamin	Dissolve 50 g sample in 15% aqueous solution Na_2SO_4 containing 1 mM NaEDTA in 250 mL volumetric flask. Filter. Clean up—Bond-Elut C_{18}	ODS-2, 5 µm, 15 cm × 4.6 mm Mobile phase–isocratic 50 mM KH_2OP_4 (pH 2.1):MeCN (90:10) Flow rate—1 mL min^{-1}	550 nm	DL—0.1 ng $injection^{-1}$ %RSD—2.7 %Recovery — 89–92	*J. Chromatogr. A*, 771, 127, 1997[98]
Standards/ cyanocobalamin	—	C_{18}, 2 or 3 µm, 37 cm × 0.075 mm Mobile phase—gradient 25 mM NH_4AC—MeCN—MeOH Flow rate—30 µL min^{-1}	ICP—MS	—	*Spectrochim. Acta, Part B*, 59, 891, 2004[99]
Foodstuffs/ cyanocobalamin	Free vitamin B_{12}—extract w/water; Total vitamin B_{12}—pepsin digestion. Clean up—immunoaffinity column	LiChrospher 100 RP18 5 µm, 25 cm × 4 mm Mobile phase—gradient Water—MeOH Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 250 nm Em λ = 312 nm	DL—3 ng g^{-1} %RSD—1–5.4 %Recovery — 95–100	*J. Chromatogr. A*, 1081, 182, 2005[100]
Food products/ cyanocobalamin	Free vitamin B_{12}—α-amylase digestion; Total vitamin B_{12}—α-amylase, pepsin digestion. Clean up—immunoaffinity column	Ace 3 AQ C_{18}, 15 cm × 3 mm Mobile phase—gradient 0.025% TFA in water (pH 2.6)–MeCN Flow rate—0.25 mL min^{-1}	UV 361 nm	%Recovery—94–100 %RSD—3.2–5.6	*J. Chromatogr. A*, 1101, 63, 2006[101]

Continued

Table 11.7 (Continued)

Sample matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
LC-MS					
Standards/cyanocobalamin	—	Kromasil C_{18}, 5 μm, 25 cm × 4.6 mm H_2O–MeCN, 0.4 mM TFA, gradient; Flow rate – 0.7 mL min^{-1}, or Zorbax Extend C_{18}, 3.5 μm, 15 cm × 2.1 mm Mobile phase—gradient H_2O–MeCN, 10 mM 1-MP Flow rate–0.2 mL min^{-1}	MS/MS-ESI Positive ion mode w/MRM or MS-ESI positive ion mode w/SIM	DL—0.2 ng mL^{-1} for Eluent I, 2 ng mL^{-1} for Eluent II	*Rap. Commun. Mass Spectrom.*, 15, 2438, 2001[102]
Fortified products, multivitamin tablets/cyanocobalamin	Free vitamin B_{12}—α-amylase digestion; Total vitamin B_{12}—α-amylase, pepsin digestion	XTerra™ MS C_{18} 5 μm, 15 cm × 3.9 mm Mobile phase—gradient Water–MeCN Flow rate—1 mL min^{-1}	MS/MS-ESI Positive ion mode w/SIM	DL—2 ng g^{-1} %RSD—2.8–3.5 %Recovery—94–98	
Pharmaceuticals					
Pharmaceutical preparations/cyanocobalamin	Shake sample w/0.05 M NaH_2PO_4. Add some chloroform to oily solutions prior to extraction w/NaH_2PO_4. Centrifuge and filter Clean up–SAX and C_{18} SPE	μBondapak C_{18}, 30 cm × 3.9 mm Mobile phase—gradient 20 mM KH_2PO_4–MeOH Flow rate—1.5 mL min^{-1}	UV 550 nm	%RSD—1.8 %Recovery—100.1	*J. Chromatogr. Sci.*, 28, 42, 1990[47]
Multivitamin—multimineral tablets/cyanocobalamin	Extract powdered tablets (10 tablets) w/extraction solution containing 0.25 g APDC, 1 g citric acid, 10 mL dimethyl sulphoxide and 30 mL water. Shake, water bath, 40 °C, 15 min. Centrifuge, dilute 15 mL supernatant w/100 mL water	μBondapak C_{18}, 10 μm, 15 cm × 3.9 mm Mobile phase – gradient H_2O–MeOH Flow rate—1.0 mL min^{-1}	UV 550 nm	%RSD—3.1–5.7 %Recovery—91–95	*J. Chromatogr.*, 541, 383, 1991[48]
Pharmaceuticals/cyanocobalamin	Dissolve or dilute sample w/water, filter	Spherisorb ODS–2, 5 μm, 15 cm × 4.6 mm Mobile phase—gradient MeOH–85 mM phosphoric acid, pH 5.2 Flow rate—1.5 mL min^{-1}	Solaar atomic absorption spectrometer 240.7 nm	DL—4.2 μg mL^{-1} %RSD—6.6 %Recovery—98.8	*Anal. Chim. Acta.*, 318, 319, 1996[49]

Multivitamin tablets/Cyanocobalamin	Sonicate one tablet w/20 mL water, 20 min, centrifuge, filter. Dilute filtrate to 25 mL w/water.	C_{18}, 5 μm, 15 cm × 4.6 mm Mobile phase—gradient H_2O–MeOH–MeCN Flow rate—mL min^{-1}	UV 362 nm	%Recovery—97–102	*Anal. Lett.*, 30, 2723, 1997[50]
Pharmaceutical preparations/Cyanocobalamin	—	MICRA NPS II (33 × 4.6 mm, 1.5 μm) Or Toso ODS (50 × 4.6 mm, 2 μm) Or Vydac C_8 (150 × 4.6 mm, 5 μm) Mobile phase—gradient 25 mM NH_4AC–MeOH–MeCN Flow rate—1 mL min^{-1}	ICP-MS	DL— 0.05–0.004 μg mL^{-1}	*J. Anal. At. Spectrom.*, 14, 1323, 1999[51]
Multivitamin tablets/cyanocobalamin	Dissolve sample in 0.1 M phosphate buffer, pH 7.0. Centrifuge and filter.	μBondapak C_{18}, 10 μm, 30 cm × 3.9 mm Mobile phase—isocratic Water:MeOH (70:30) Flow rate—0.8 mL min^{-1}	Fluorescence Ex λ = 275 nm Em λ = 305 nm	DL—0.1 ng mL^{-1} %RSD—1.8–4.1 %Recovery—94–102	*J. Chromatogr. A*, 891, 243, 2000[52]
Multivitamin tablets/Cyanocobalamin	Sonicate 10 tablet w/350 mL MeOH-water (50:50), 30 min, filter. Dilute 25 mL filtrate w/50 mL same solvent.	Phenylpropanolamine, 15 cm × 4.6 mm Mobile phase—isocratic 30 mM phosphate buffer (pH 3):MeCN (94:6) Flow rate—1 mL min^{-1}	UV 361 nm	DL—25 ng %RSD—0.5 %Recovery—100	*J. Chromatogr. A*, 870, 217, 2000[53]
LC-MS					
Pharmaceutical preparations/cyanocobalamin	—	Brownlee Aquapore C_{18} 7 μm, 10 cm × 1 mm & Vydac C_8 5 μm, 15 cm × 1 mm Mobile phase—gradient 25 mM acetate in water (pH 4)–MeOH Flow rate—40 μL min^{-1}	MS-ESI positive ion mode w/SIM or MS/MS-ESI positive ion mode w/MRM	DL—5–10 ng mL^{-1}	*Anal. Chim. Acta.*, 359, 227, 1998[104] *Analyst*, 123, 131, 1998[105]
Biologicals					
Standards/Cyanocobalamin	—	μBondapak C_{18}, 30 cm × 3.9 mm Mobile phase—isocratic H_2O:HAC:IPA (90:1:9) Flow rate—0.3 mL min^{-1}	UV 365 nm	—	*Anal. Biochem.*, 125, 253, 1982[106]

Continued

Table 11.7 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Biologicals (*Continued*)					
Cells/cyanocobalamin	Wash L1210 cell w/phosphate-buffered saline, add 80% EtOH extract at 70 °C, 20 min (80°C, 30 min for *L. leichmannii* cells). Centrifuge, remove cell debris, extract w/EtOH again, combine the extracts, and evaporate to dryness. Dissolve in water. Clean up—Sep-Pak C_{18}	LiChrosorb C_8, 10 μm, 25 cm × 4.6 mm Mobile phase gradient or Isocratic—30% MeCN in 1 mM NH_4AC, pH 4.4 or Gradient—0.05 M H_3OP_4 (pH 3)–MeCN Flow rate—2 mL min^{-1}	UV 254 nm	—	*Anal. Biochem.*, 120, 394, 1982[107]
Bacterial cells/ cyanocobalamin	Resuspend frozen cells in 10 volumes water and ajust pH to 5–6 using HAC. Mix suspension w/KCN at room temperature. Incubate at 100°C, 20 min. Centrifuge, reextract pellet. Combine supernatants Clean up—XAD-4 column.	μBondapak C_{18}, 30 cm × 3.9 mm Mobile phase—gradient MeOH—HAC (0.1%, v/v) Flow rate—1 mL min^{-1}	UV/VIS 254 nm and 546 nm	%Recovery—75–80	*Anal. Biochem.*, 155, 365, 1986[108]
Human plasma/ cyanocobalamin	Dilute plasma sample in 1% HAC and centrifuge. Purify and enrich the dilute sample on C_{18}	C_{18} ODS-2, 5 μm, 30 cm × 4.6 mm Mobile phase—isocratic NaH_2PO_4 (0.1 M, pH 4.0):MeOH (63:27) Flow rate—0.8 mL min^{-1}	UV 361 nm	%RSD—3.5 %Recovery—97	*J. Chromatogr.* B, 667, 129, 1995[109]
Biological samples/cyanocobalamin	Homogenize sample w/10 mM KH_2PO_4 (pH 7), centrifuge. Filter	Discovery RP-Amide C_{16}, 5 μm Mobile phase—gradient 10 mM KH_2PO_4 (pH 7):MeCN Flow rate—1 mL min^{-1}	UV 361 nm	DL—15 ng mL^{-1} QL—51 ng mL^{-1} %RSD—1.3 %Recovery—95–99	*Chromatographia*, 58, 5, 2003[110]

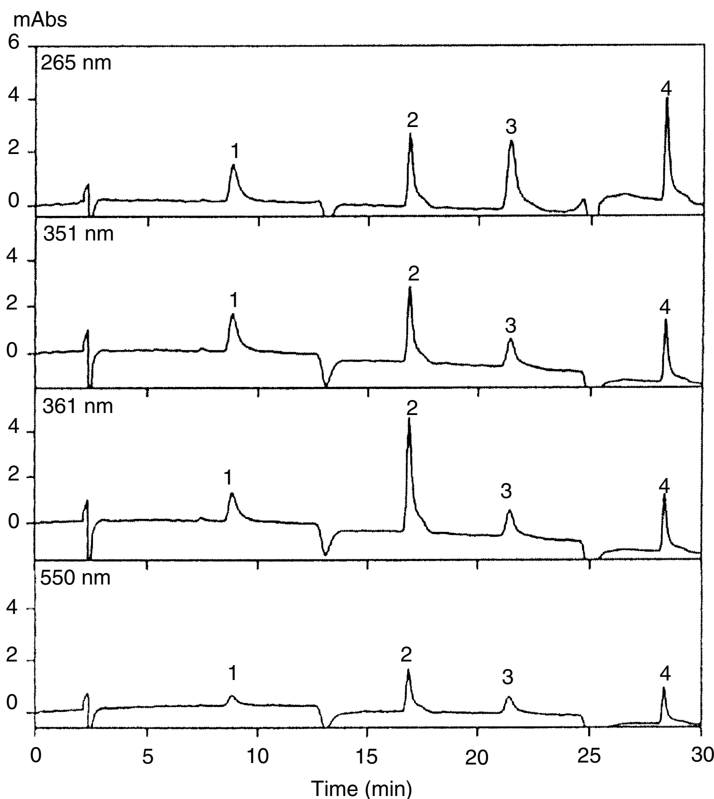

Figure 11.7 Chromatographic profile using the amide-based column and the recommended elution program. Flow-rate, 1 mL min^{-1}; injected sample (containing 0.5 µg mL^{-1} of each cobalamin), 10 µL. The peaks correspond to: 1 = OH-Cbl; 2 = CN-Cbl; 3 = Ado-Cbl; 4 = Me-Cbl. (Reproduced from Viñas, P., López-Erroz, C., Balsalobre, N., and Hernández-Córdoba, M., *Chromatographia*, 58, 5, 2003. With permission.)

the need for instrumentally based assays, and early methods relied on off-line isotope dilution or radio-ligand binding to provide sensitivity and, to some degree, specificity.[45,46]

It is recognized that improved LC-MS instrumentation and LC interfaced with inductively coupled plasma emission mass spectrometry (LC-ICP-MS)[51,99,104] yield highly sensitive and specific methods for cobalamins. Yanes and Miller-Ihli[99] showed that CNCbl, OHCbl, AdoCbl, and MeCbl were well resolved by LC-ICP-MS with clean chromatographs produced by the ICP-MS due to the element specificity (Figure 11.8). The method was rugged and applicable to assay of foods and supplements. Matrix interference was minimized. This method is detailed in Section 10.4.

Only a few methods are available that take advantage of LC-MS technology.[102–105] Chassaigne and Łobiński[104] compared detection modes including spectrophotometric, ICP-MS, and electrospray ionization mass spectrometry (MS-ESI) coupled to microbore reversed-phase LC on C_8. Using standards of CNCbl, AdoCbl, OHCbl, MeCbl, and cobinamide dicyanide $(CN)_2Cb_n$, ICP-MS was more sensitive than any other online detection system; however, total ion current chromatograms allowed unambiguous species identification. Chassaigne and Łobiński[105] also showed that MS-ESI or tandem ESI (MS/MS-ESI) provided a direct species-selective assay of cobalamins in pharmaceuticals. Recent work by Luo et al.[103] expanded LC-MS-ES to analysis of fortified foods. Mass spectrometry in the selected ion recording (SIR) mode at m/z 930.8 was used for quantitation. Alsberg et al.[102] used LC-MS/

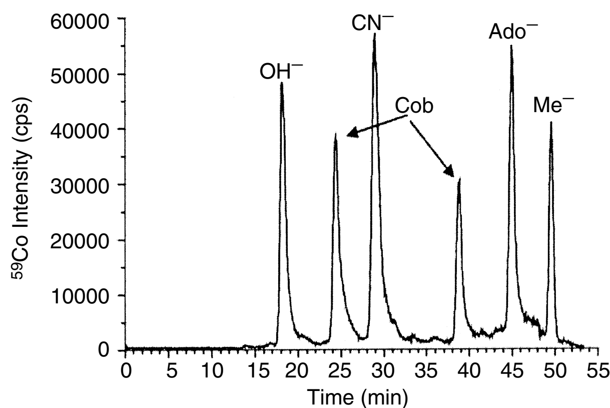

Figure 11.8 Chromatogram for optimized reversed-phase μHPLC-ICP-MS (C_{18}, 12 μm). Gradient Elution Program: 50% C, 15 min; 50%–90% C, 18 min; 90% C, 3 min; 90%–50% C, 2 min; 50% C, 3 min. (Reproduced from Yanes, E. G. and Miller-Ihli, N. J., *Spectrochim. Acta Part B*, 59, 891, 2004. With permission.)

MS-ESI with SIR to quantify OHalkyl-Cbl_2. Limits of detection and limits of quantitation were 0.2 and 2 pg μL^{-1} for α-hydroxy propylcobalamin. The authors stated that the low detection levels make the procedure useful for *in vivo* levels. Undoubtedly, application of LC-MS to vitamin B_{12} analysis of complex matrices is at its infancy.

11.3.3.4 Optical biosensor protein-binding assay

Indyk et al.[111] evaluated the Biacore International Vitamin B_{12} Qflex kit for assay of vitamin B_{12} in milk products, infant formulas, meats, and liver. The Biacore procedure is based upon a biosensor-based, nonlabeled inhibition protein-binding assay using nonintrinsic R-protein. The R-protein (haptocorrin) was selected as the protein binder due to its affinity for corrinoids and higher degree of stability in solution compared to the intrinsic factor.[111] The Biacore Q^R methodology is a bimolecular interaction analysis using surface plasmon resonance that detects nonlabeled interaction of specific binding with an immobilized ligand on a carboxymethyl dextran-modified gold sensor surface. The detector measures changes in the resonance angle due to the molecular interaction at the surface. The Biacore International instrumentation has been successfully used for the assay of folic acid (Chapter 10), biotin (Chapter 12), and pantothenic acid (Chapter 13). For these vitamins, the assays were immuno-based with monoclonal antibodies; however, for vitamin B_{12}, hapten-specific vitamin B_{12} antibodies are difficult to form.[111]

For the different foods included, the study showed that the vitamin B_{12} Qflex kit provided by Biacore International produced results comparable to microbiological assay by *L. delbruekii* and by an isotope-labeled protein-binding assay. Validation parameters were excellent with HORRAT values ranging from 0.13 to 0.70. The authors of the study characterized the Biacore International biosensor procedure as a robust and practical alternative to established analysis methods. Similar observations were made for the biosensor methods for folic acid, biotin, and pantothenic acid.

11.4 Method protocols

Cobalamin Speciation Using Reversed-Phase Micro-High-Performance Liquid Chromatography Interfaced to Inductively Coupled Plasma Mass Spectrometry
Spectrochim. Acta B, 59, 891, 2004[99]

Principle
Micro LC-ICP-MS was optimized for resolution and speciation of CNCbl, OHCbl, MeCbl, and AdoCbl. Gradient elution on a C_{18}, 3 µm column, 30 cm × 75 µm i.d. resolved cobalamins (CNCbl, OHCbl, MeCbl, and AdoCbl). ICP-MS provided excellent specificity and sensitivity.

Chemicals

- CNCbl
- OHCbl
- MeCbl
- AdoCbl
- MeOH
- Acetonitrile
- Ammonium acetate

Apparatus

- Liquid chromatograph
- ICP-MS

Chromatography

Column	30 cm × 75 µm
Stationary phase	Unimicro C_{18}, 2 or 3 µm
Mobile phase	Gradient ammonium acetate/MeOH/acetonitirile
Injection volume	20 nL
Detection	ICP-MS

HPLC-ESI-MS Analysis of Vitamin B_{12} in Food Products and in Multivitamins—Multimineral Tablets
Anal. Chim. Acta, 562, 185, 2006[103]

Principle
Assay includes enzyme digestion for extraction and LC-ESI-MS.

Chemicals

- α-Amylase
- Pepsin
- MeOH
- Sodium cyanide

Extraction of Food

- Weigh 15–30 g into Erlenmeyer
- Add 40 mL 50 mM sodium acetate buffer, pH 4.0, 1 mL of 1% sodium cyanide (1.0%), 0.25 g α-amylase, 1 g pepsin
- Digest at 37°C for 3 h
- Cool, adjust pH to 4.8
- Heat, 100°C, 35 min
- Cool, dilute to volume with water
- Shake, centrifuge, filter (0.45 µm membrane)

Apparatus

- Liquid chromatograph
- ESI-MS

Chromatography

Column	15 cm × 3.9 mm
Stationary phase	×Terra MS
Mobile phase	Gradient, water/acetonitrile
Column temperature	Ambient
Flow rate	1 mL min^{-1}
Detection	ESI-MS, positive ion mode

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chapter twelve

Biotin

12.1 Review

Wildiers (1901) reported the presence of a growth factor in yeast and meat extracts referred to as "bios," which was required for yeast growth.[1] Bios was fractionated over the next decades into bios I (meso-inositol), bios II (an impure preparation of pantothenic acid), and bios II B (later identified as biotin). Recognition of the toxic effects of raw egg white in the animal diet occurred before the turn of twentieth century and was further defined in 1916 by Bateman. Boas[2] (1927) confirmed earlier descriptions of egg white injury in rats fed raw egg white and showed the curative effects of raw liver. In 1936, Kögl and Tonnis[3] isolated and crystallized a growth factor from egg yolk, which they named biotin. Snell, in 1941, reported that the glycoprotein "avidin" was the biotin antagonist responsible for the toxicity of raw egg white.[4] The structure was determined in 1942 with synthesis in 1943.

Biotin deficiency is characterized by anorexia, nausea, vomiting, glossitis, pallor, mental depression, hair loss (alopecia), dry scaly dermatitis, and an increase in serum cholesterol and bile pigments.[5] Human deficiency is rare but can occur through prolonged consumption of raw egg and parenteral feeding without biotin supplementation in patients with short-gut syndrome.[5,15] Biotin deficiency occurs in infants through an inborn error of metabolism that leads to lack of biotinidase, the enzyme necessary to cleave biotinyl-lysine bonds in biocytin (biotinyl-ε-lysine). Mock[5] indicates that the lack of biotinidase produces biotin deficiency by leading to inefficient release of protein-bound biotin in the gastrointestinal tract, less salvage of biotin at the cellular level, and through increased renal loss. Biotin deficiency has been linked to sudden infant death syndrome; however, firm conclusions have not been made in this significant area. Biotinidase deficiency results in a secondary biotin deficiency owing to inefficient biotin absorption in the gut, less salvage of biotin at the cellular level, and increased renal loss of biotin and biocytin.[6] Profound biotinidase deficiency is defined by a less than 10% level of normal serum activity. Symptoms of biotinidase deficiency include seizures, hypotonia, skin rash, alopecia, developmental delay, conjunctivitis, visual problems, hearing problems, mental retardation, keetolactic acidosis, and organic aciduria.[7–9] More accurate assay techniques primarily using liquid chromatography (LC) linked to avidin-binding assays have provided better status indicators. Measurement of urinary excretion and excretion of biotin metabolites are, now, good indicators of biotin status.[6,10–12] Decreased urinary excretion of biotin and bisnorbiotin are early and sensitive indicators of biotin deficiency while serum concentration change is not.[10,11] Urinary excretion of 3-hydroxyisovaleric acid, a leucine metabolite, increases owing to the lack of β-methylcrotonyl-CoA carboxylase due to biotin deficiency. Increased urinary excretion of this metabolite is considered to be a good indicator of biotin deficiency.[6,11] Assessing

marginal biotin deficiency is of particular concern during pregnancy since the deficiency is teratogenic in some animal species.[12–14]

Biotin occurs in relatively low concentrations in most foods. Liver and yeast are high in biotin content compared to other foods. Substantial sources are egg yolk, dairy products, soy products, cereals, and vegetables. Meat and fruit are low in biotin.[15] Data on the biotin content of food is not published in most food composition databases.[15] Relatively few studies have been published with few foods analyzed.[16–24] More recent development of sensitive and specific LC/avidin binding assays offer improved analytical values for food.[24] Staggs et al.[24] used LC coupled to an off-line avidin binding assay that distinguishes biotin from inactive metabolites to assay total biotin content of 87 foods. Comparison of the values to previously published values showed that the values obtained with the LC/avidin binding methods disagreed substantially with previously published values for like foods. Values varied from 247 times greater than published to as much as 36% less. Among 51 foods assayed for which published values were available, only seven agreed within ±20%. The authors concluded that most published values for biotin are likely inaccurate. Data from the Staggs et al.[24] study are provided in Table 12.1. Much of the previously published data was obtained by microbiological assay with *Lactobacillus plantarum* ATCC 8014. Biotin is present in food in the free form and covalently bound to polypeptides through a lysine residue.[6] Proteases degrade the protein-bound biotin to biocytin (biotinyl-ε-lysine), which is broken down to liberate free biotin by biotinidase, which facilitates absorption. Microbial synthesis in the gut is a source of biotin; however, the significance of microbial synthesis to total biotin available to the human is unknown. The role of avidin as a biotin antagonist is understood. Nutritionally, the binding phenomenon has little impact since cooking destroys the avidin–biotin complex and denatures avidin, preventing additional complex formation. Raw egg white, if added to foods without further cooking or ingested with cooked foods, provides avidin that binds the low amounts of biotin in the food. The avidin–biotin complex resists digestive proteases and prevents absorption.

Recommended Dietary Allowances (RDAs) were not set by the Dietary Reference Intake (DRI) committee.[15] Adequate intakes (AIs) range from 5 $\mu g\ d^{-1}$ for infants (0–6 months) to 35 $\mu g\ d^{-1}$ for lactating women (Table 12.2). Dietary intake has been estimated at 39.9 ± 26.9 $\mu g\ d^{-1}$ for women aged 18–24 years;[15] however, taking the Staggs et al.[24] study into account, such intake data can be considered suspect. The Reference Daily Intake (RDI) for use in calculation of nutrition label information is 300 μg.[25]

Metabolically, biotin is the coenzyme for carboxylases that incorporate bicarbonate into various substrates. Mammalian carboxylases are acetyl-CoA carboxylase (fatty acid synthesis), pyruvate carboxylase (oxaloacetate formation), 3-methylcrotonyl-CoA carboxylase (leucine degradation), and propionyl-CoA carboxylase (methylmalonyl-CoA formation).[26] Other carboxylases are known to exist in the plant kingdom.[27,28] Noncarboxylase roles for biotin are now becoming evident. Specific roles for biotin include cell signaling, gene expression, and site specific modifications of histones that affect chromatin structure.[29–31]

12.2 Properties

12.2.1 Chemistry

12.2.1.1 General properties

Biotin is *cis*-hexahydro-2-oxo-1H-thieno [3,4-d] imidazole-4-pentanoic acid.[32] Eight stereoisomers exist. d(+) Biotin is the only biologically active form and other isomers are not found in nature.[33,34] The biotin structure is shown in Figure 12.1. The bicyclic ring structure

Table 12.1 Biotin Content of Various Foods

Food	μg 100 g^{-1}
Meat, fish, poultry, egg	
Chicken liver, cooked	187.2
Beef liver, cooked	41.6
Egg, yolk, cooked	27.2
Egg, whole, cooked	21.4
Salmon, pink, canned in water	5.9
Egg, white, cooked	5.8
Hamburger patty, cooked	4.5
Pork chop, cooked	4.5
Hot dog, chicken and pork, cooked	3.7
Chicken nuggets, breaded, fried	1.3
Fish sticks, minced, breaded, fried	1.0
Catfish, breaded, fried	0.7
Sliced turkey, processed deli	0.7
Tuna, canned in water	0.7
Chicken strips, breaded, fried	0.4
Dairy	
American cheese	3.1
Cheddar cheese, mild	1.4
Chocolate milk, low-fat	0.4
Plain yogurt	0.1
Provolone cheese	0.1
Skim milk	0.1
2% Milk	0.1
Whole milk	0.1
Cereals	
Cheerios®	0.1
Frosted Flakes®	0.1
Golden Grahams®	0.1
Kix®	0.1
Vegetables	
Mushrooms, canned	2.2
Sweet potato, cooked	1.5
Broccoli, fresh	0.9
Spinach, frozen	0.7
Carrots, canned	0.6
Cauliflower, fresh	0.2
Green beans, canned	<0.1
Whole kernel corn, canned	<0.1
Fruits and berries	
Strawberries, fresh	1.5
Avocado, fresh	1.0
Tomatoes, fresh	0.7
Orange juice, canned, from concentrate	0.4
Raisins	0.4
Raspberries, fresh	0.2
Apple juice, canned, from concentrate	0.1
Banana, fresh	0.1
Apple, fresh	<0.1
Orange, fresh	<0.1

Continued

Table 12.1 (Continued)

Food	μg 100 g^{-1}
Bread and grains	
Grilled toast	1.2
Crackers, saltine	0.3
Hamburger bun	0.3
Noodles	0.2
Oatmeal	0.2
Grits	0.1
Whole wheat bread	0.1
Roll, dinner	<0.1
Sweets	
Banana pudding	1.0
Sugar cookie	0.3
Chocolate sandwich cookie	0.1
Oatmeal cream pie	0.1
Poptart, blueberry	<0.1
Vanilla cake with frosting	<0.1
Entrees	
Pepperoni pizza	2.1
Chili	0.5
French fries	0.3
Salad, mixed garden	0.3
Hush puppies	0.2
Beef-vegetable soup	0.1
Cheese pizza	0.1
Corn chip chili pie	0.1
Macaroni and cheese	0.1
Mashed potatoes, with brown gravy	0.1
Ramen noodles, oriental	0.1
Tator tots	0.1
Condiments and sauces	
Mayonnaise	0.2
Ranch dressing	0.2
Ketchup	0.1
Spaghetti sauce, with beef	0.1
Beverages	
Red fruit punch	0.2
Beer	0.1
Coke®	0.1
Tea, sweet	0.1
White wine	0.1
Nuts	
Peanuts, roasted, salted	17.5
Sunflower seeds, roasted, salted	7.8
Almonds, roasted, salted	4.4
Walnuts, fresh	2.6
Pecans, fresh	2.0
Miscellaneous	
Yeast	20.2
Potato chips, baked, barbecue flavor	0.1

Source: Staggs, C. G., Sealey, W. M., McCabe, B. J., Teague, A. M., and Mock, D. M., Determination of biotin content of select foods using accurate and sensitive HPLC/avidin binding, *J. Food Compos. Anal.*, 17, 767, 2004.

Table 12.2 Dietary Reference Intakes for Biotin

Life stage	DRI μg d^{-1}
Infants (months)	
0–6	5
7–12	6
Children (years)	
1–3	8
4–8	12
Males (years)	
9–13	20
14–18	25
19–30	30
31–50	30
51–70	30
>70	30
Females (years)	
9–13	20
14–18	25
19–30	30
31–50	30
51–70	30
>70	30
Pregnancy (years)	
≤18	30
19–30	30
31–50	30
Lactation (years)	
≤18	35
19–30	35
31–50	35

Ordinary type: adequate intake (AI).

Source: Wolf, B., Biotinidase: its role in biotinidase deficiency and biotin metabolism, *J. Nutr. Biochem.*, 16, 441, 2005.

contains a ureido ring, which is fused to a tetrahydrothiophene ring with a valeric acid side chain. Binding with carboxylase enzymes is through an amide linkage between the carboxyl group of biotin and an ε-amino group of the enzyme protein. Biocytin (Figure 12.2) is formed by proteolysis of the biotin–enzyme complex. Biotin is released from biocytin by biotinidase to conserve biotin in the cellular pool.

In nature, biotin occurs free or bound through ε-amino linkages with lysine as biocytin or to carrier proteins and peptides. It exists in the free state in plants to a greater extent than in animal tissues.[32,35] Mock et al.[21] reported that greater than 95% of the biotin in human milk was present as free biotin. Scheiner[36] showed that various animal-based feed ingredients contained from 11% to 29% free biotin; whereas, cereal and plant ingredients contained from 23% to 80% free biotin. Because of the bound nature, extraction procedures to measure total biotin must liberate the vitamin. Complexity of the binding systems in foods promotes variability in biological availability owing to differential susceptibilities of the biotin–protein linkages to digestion.[37]

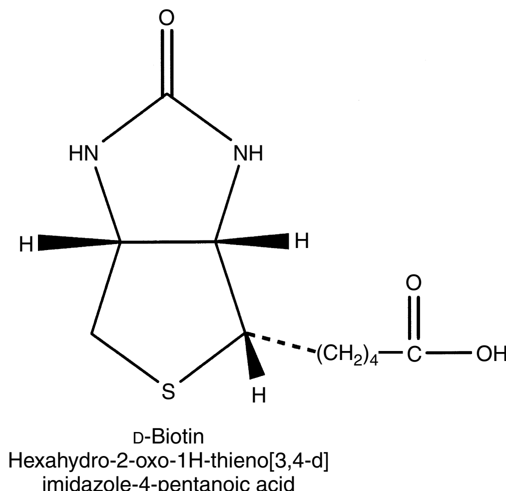

Figure 12.1 Structure of biotin.

Physical properties of biotin are given in Table 12.3. The United States Pharmacopeial Convention (USP) standard is d-biotin. Formulations for use by the pharmaceutical and food industry and for the animal feed industry include crystalline biotin, combination premixes, and with diluents such as dicalcium phosphate to aid in dispersibility and ease of blending. It is soluble in alkali solutions but sparingly soluble in water (20 mg/100 mL at 25°C) and 95% ethanol. It is insoluble in organic solvents.

12.2.1.2 Spectral properties

Biotin has weak absorbance at 200–220 nm with an absorption maxima at 204 nm from the carbonyl group.[38] LC methods are only useful for assay of highly concentrated pharmaceuticals or vitamin premixes unless UV absorbance or fluorescence is enhanced through derivatization. Such approaches are discussed in Section 12.2.3.3.

12.2.2 Stability

The sulfur atom in biotin is subject to oxidation with the sequential formation of biotin sulfoxide and biotin sulfone (Figure 12.2). The susceptibility of sulfur to oxidation provides a primary route for loss of biotin activity in processed foods. Oxidation produces a mixture of l- and d-biotin sulfoxides. Further oxidation to the sulfone leads to complete loss of biological activity. Humans have limited capability to reduce d-biotin sulfoxide back to d-biotin. β-Oxidation of the valeric acid side chain yields bisnorbiotin and tetranorbiotin (Figure 12.2). Susceptibility to sulfur oxidation and β-oxidation can produce degradation; however, losses in foods have not been well defined under specific processing and storage conditions. This is due, in part, to the fact that deficiencies have not been commonly diagnosed, and dietary supplies have been considered sufficient. Of those processing studies that have been published, Teaquel et al.[40] showed that biotin was stable in frozen foods at 20°C over a 2-month storage period. Likewise, biotin in legumes was not subject to leaching loss by presoaking treatments and cooking.[41] Biotin differs in this respect from most other water-soluble vitamins that are quite prone to leaching losses. The fact that a high proportion of biotin in vegetables is protein-bound most likely decreases leaching into the

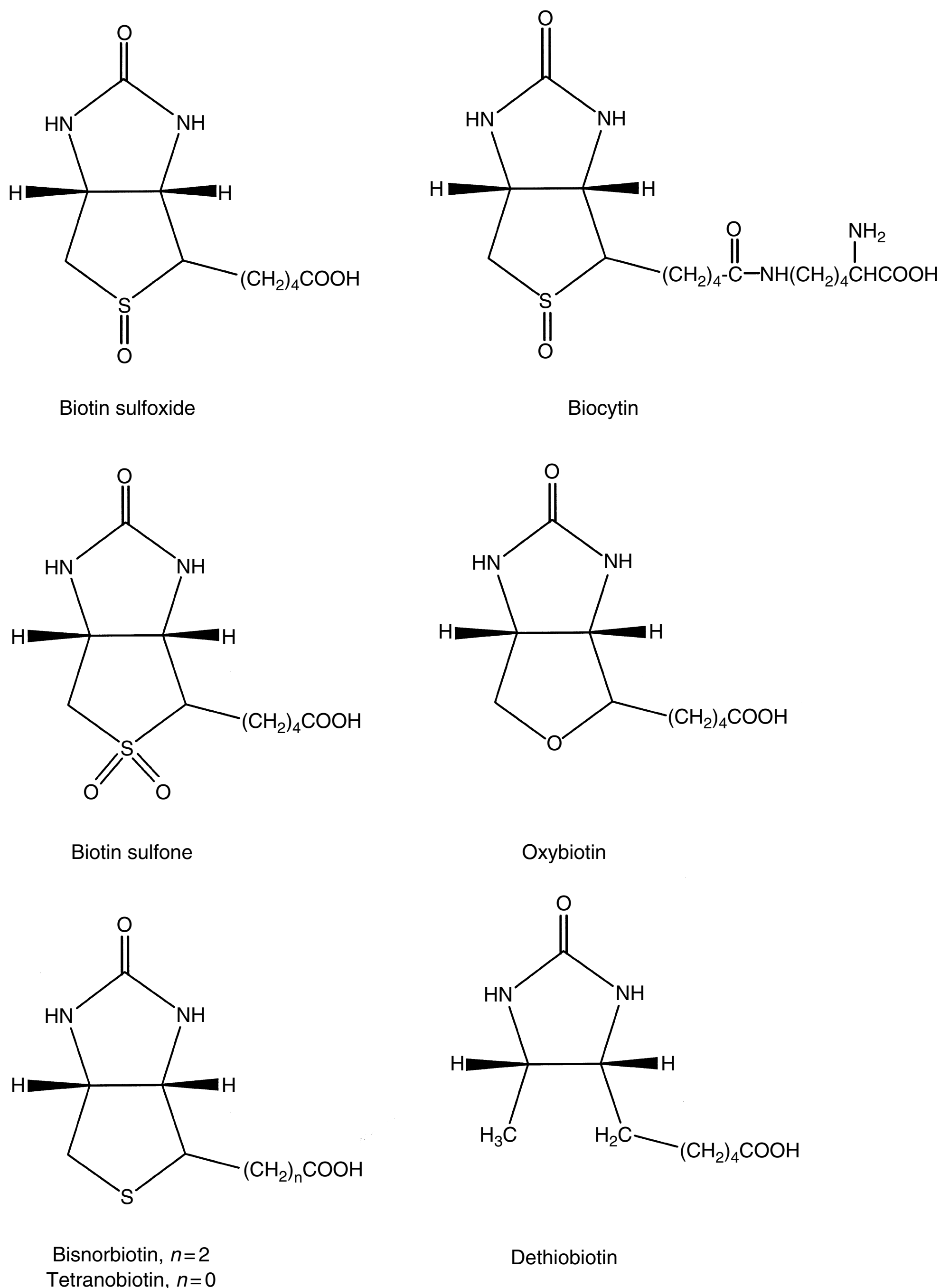

Figure 12.2 Structures of biotin metabolites and analogs.

cooking medium. As biochemists and nutritionists learn more about biotin metabolism and various disease state relationships, a better understanding of processing effects might be required.

Biotin in solution is quite stable at pH 4.0–9.0.[38] Biotin is commonly extracted by autoclaving biological samples in 2 *N* or 6 *N* sulfuric acid for 2 h (Section 12.2.3.1).

Table 12.3 Physical Properties of Biotin

Substance[a]	Molar mass	Formula	Solubility	Melting point °C	Crystal form	Absorbance λ_{max} (nm)
Biotin CAS No. 58-85-5 **1272**	244.31	$C_{10}H_{26}N_2O_3S$	Soluble in dilute alkali Sparingly soluble in water, alcohol Insoluble in most organic solvents	232–233	Colorless, fine long needles	204 Very weak

[a] Common or generic name; CAS No.: Chemical Abstract Service number, bold print designates the Merck Index monograph number.

Source: Ball, G. F. M., Chemical and biological nature of the water-soluble vitamins, In *Water-Soluble Vitamin Assays in Human Nutrition*, Chapman & Hall, New York, 1994, chap. 2.; Budavari, S., *The Merck Index*, 13th ed., Merck and Company, Whitehouse Station, NJ, 2001, 1231.

12.2.3 Bioavailability

Bioavailability of biotin largely depends on the ability of proteases and peptidases available in the gastrointestinal tract to convert the protein-bound biotin to biocytin which, in turn, is converted to free biotin by biotinidase.[42,43] Since this process is essential, dietary factors, such as ingestion of biotin-binding proteins like avidin, can decrease bioavailability. However, such binding proteins are believed to be rare in the food supply with eggs being somewhat unique in this respect. Since the bioavailability of biotin in the human is estimated from recovery of biotin in urine, assays that do not measure biotin plus metabolites underestimate biotin bioavailability.[10] Quite recent development of the LC/avidin-binding assay that is specific for biotin and biotin metabolites allows more exact measurement of bioavailability.[10,44] Zempleni and Mock[44] showed that biotin provided at pharmacological dosages was 100% bioavailable as measured by the LC/avidin-binding assay. Biotin in the urine was present as free biotin (>50%), bisnorbiotin (13%–23%), biotin-d,l sulfoxide (5%–13%), bisnorbiotin methyl ketone (3%–9%), and biotin sulfone (1%–3%). Bioavailability from dietary sources has not been investigated for humans.

12.3 Methods

Biotin was assayed by microbiological methods for many decades. Avidin-binding assays developed in the 1970s were substantially based on the work of Hood.[45–47] Initial methods were based upon isotope dilution assays; however, many modifications adapting the strong and specific binding of avidin with biotin incorporate immunological and enzyme protein binding assay (EPBA) approaches. Most clinical studies and research on biological tissues and fluids have relied heavily on the avidin-binding assays. Conversely, such specific and highly sensitive methods have not been adapted to food studies. Almost all available data on the biotin content of the food supply has been obtained by assay with *L. plantarum* ATCC 8014. LC methods can efficiently separate biotin, the sulfoxides, sulfones, and other biotin analogs. Commonly available LC detectors cannot be used for direct quantitation for biotin owing to its low, nonspecific absorbance and lack of fluorescence. LC methods coupled with avidin-binding detection, formation of fluorescent derivatives, and other approaches have been developed and used effectively to dramatically improve quantitation of biotin and its metabolites in serum and urine. These methods are discussed in this section. This methodology has not been extensively used for food assay, but there is no reason that LC coupled with

Table 12.4 Regulatory and Handbook Methods for Analysis of Biotin

Source	Form	Methods and application	Approach	Most current cross-reference
***U.S. Pharmacopeia National Formulatry**, USP 29/NF 24, Dietary Supplements Official Monographs, 2006*[57]				
1. Pages 2392, 2395, 2398, 2403, 2413–2415	Biotin	Biotin in water-soluble capsules/ tablets w/wo mineral, oral solution w/mineral	LC 200 nm or Microbiological	None
2. Pages 2427, 2430, 2431, 2436	Biotin	Biotin in water-soluble capsules/tablets w/wo minerals	LC 200 nm or Microbiological	None
3. Pages 283–284	Biotin	Biotin (NLT 97.5%, NMT 100.5%)	Titration, Sodium hydroxide	None
***Food Chemicals Codex**, 5th ed., 2004*[58]				
1. Pages 49–50	Biotin	Biotin (NLT 97.5%, NMT 100.5%)	Titration, Sodium hydroxide	None
***British Pharmacopeia**, 2007*[59]				
1. Pages 269–270	Biotin	Biotin (NLT 98.5%, NMT 101.0%)	Titration	None

newer detection approaches would not add a new dimension to biotin determination in foods.

Useful reviews on biotin analysis include Scheiner,[35] Bowers-Kömro et al.,[48] Stein et al.,[49] Eitenmiller and Landen,[50] Mock,[51] Hentz and Bachas,[52] Rehner and Stein,[53] Lizano et al.,[54] Huang and Rogers,[55] Shiuan et al.,[56] Ploux,[33] Mock,[34] and Livaniou et al.[12] These reviews, particularly with the availability of the 1997 articles in *Methods of Enzymology*, vol. 279,[51–55] provide extensive and current sources for procedures for biotin assay. Table 12.4 provides a summary of regulatory and handbook methods for biotin assay. High performance liquid chromatography (HPLC) methods of the USP are only applicable to highly concentrated pharmaceuticals or pure biotin. Association of Official Analytical Chemists (AOAC) International[60] and the American Association of Cereal Chemists[61] do not provide a method for biotin. The AOAC International Task Force on Methods for Nutrition Labeling recommended that a microbiological assay by *L. plantarum* ATCC 8014 be considered for collaborative study.[62] At present, collaborative studies have not been completed.

12.3.1 Microbiological methods

Traditionally, biotin in foods and other biological samples was quantitated by microbiological assay with *L. plantarum* ATCC 8014. The *L. plantarum* assay was originally introduced by Wright and Skeggs in 1944.[63] Less frequently used microorganisms include *L. casei ssp. rhamnosus*, *Saccharomyces cerevisiae*, *Neurospora crassa*, *Ochramonas danica*,[64] *Escherichia coli* KS302bio,[65] and *Kloeckera apiculata (brevis)*.[66,67] *L. plantarum* is more specific for biologically active forms of biotin than other biotin requiring microorganisms.[35] It does, however, respond to dethiobiotin, which spares biotin and can cause significant overestimation of the biotin content, if present in sample extracts.[68] Langer and György[69] reviewed early work on microbial responses to biotin and related compounds. Growth response characteristics of *L. plantarum* include

1. Lack of response to bound forms of biotin. Biocytin must be cleaved by extraction conditions, usually by autoclaving in 2 *N* or 6 *N* sulfuric acid.

2. Equal growth response to biotin sulfoxide, but no response to biotin sulfone.
3. Fatty acids can stimulate growth. Lipids should be extracted before acid hydrolysis to avoid fatty acid effects.[36]

Details of the *L. plantarum* assay were provided by Scheiner.[35,36] Extraction procedures by product matrix include the following:

- ***Tablets and Capsules***: Homogenize in 0.1 *N* sodium hydroxide. Dilute and centrifuge to clarify. Dilute and adjust the final dilution to pH 6.8. The assay solution should approximate 0.2 ng/mL in concentration.
- ***Granulations and Premixes***: Mix with 0.1 *N* sodium hydroxide. Swirl for 10 min, dilute, and centrifuge. Make final dilution (0.2 ng/mL), adjust to pH 6.8.
- ***Pure Biotin***: Add 1 mL of 0.1 *N* sodium hydroxide for each 10 mg. Dissolve by swirling. Transfer to 1 L volumetric flask and bring to volume. Dilute an aliquot to proper assay range (0.2 ng/mL).
- ***Solutions and Suspensions***: Pipet an aliquot into volumetric flask, bring to volume. Dilute aliquot to the proper assay range (0.2 ng/mL). For suspensions, it may be necessary to add a few drops of 1 *N* sodium hydroxide to the first dilution to ensure complete solubilization of biotin.
- ***Foods and Feeds***: To 5 g of sample, add 2 *N* sulfuric acid to plant products and 6 *N* sulfuric acid to animal products. The ratio of acid to sample should be 20:1. Autoclave at 121°C for 2 h. Cool, dilute with water, filter or centrifuge an aliquot, adjust pH to 6.8 with 20% sodium hydroxide, and dilute to the proper assay range (0.2 ng/mL).

Note: High fat samples should be extracted with hexane or ethyl ether to avoid fatty acid stimulation of the growth response.

The standard curve range is 0.025–2.5 ng biotin per 10 mL volume in the assay tube.

Few modifications have been made to the standard biotin assay by *L. plantarum*. However, an innovative radiometric assay with *K. apiculata* (brevis, ATCC 9774) was introduced by Guilarte[66,67] for analysis of plasma and foods. $^{14}CO_2$ produced by the metabolism of L-1-[^{14}C]-methionine in the presence of biotin was measured. CO_2 production was proportional to the amount of biotin present in the growth medium. *K. apiculata* did not respond to dethiobiotin, dehydrobiotin, and biotin sulfone. Biotin-d-sulfoxide was as active as biotin, but biotin-L-sulfoxide was only 10%–15% as active. Unlike *L. plantarum, K. apiculata* responds to intact biocytin. *K. apiculata* response was not affected by fatty acids and aspartic acid, which can stimulate growth of *L. plantarum*. For plasma analysis, *K. apiculata* values were lower than those obtained by *L. plantarum*. Guilarte[66] attributed the higher values obtained by the *L. plantarum* assay to plasma fatty acids that spared the organism's biotin requirement. For food products,[67] the radiometric assay gave comparable data to the *L. plantarum* assay except for cereals. For all cereal samples, the radiometric assay indicated higher content of biotin compared to the *L. plantarum* assay. The research showed that biotin was removed from the sample extract by filtering, after acid hydrolysis. Filtration is commonly used to clarify sample extracts before microbiological assay and is not necessary for the radiometric assay. Although the *K. apiculata* radiometric procedure was shown to have advantages of better specificity, easier sample preparation, and other time-saving attributes, it has not been routinely used for food analysis. Use of radioisotopes has been mentioned by several authors as undesirable in food analysis laboratories. Stricter regulations and higher disposal costs make their use more difficult today, compared to the time when the methods were originally developed.

12.3.2 Avidin-binding assays

The ability of avidin to stoichometrically bind to biotin in a stable complex is the basis of current methods to quantitate biotin and certain metabolites from biological materials.

Radioligand binding assays were initially developed by Dakshinamurti et al.[70] and Hood[45–47] using ^{14}C-biotin. Yankofsky et al.[71] modified the ^{14}C-based assay with the introduction of solid-phase binding assay that used covalently linked avidin to uniform cellulose disks. During the same time period, an avidin-binding assay with [^{3}H]-biotin was reported.[72–74] Later modifications include the introduction of ^{125}I-biotin[75] and ^{125}I-biotin ^{3}H-biotin derivatives combined with double antibody techniques to separate free ligand from bound ligand.[76,77] Further modification of radioligand binding procedures was the introduction of solid-phase assays.[78–83] Of the various modifications of radioligand binding methods, the sequential solid-phase assay developed by Mock and DuBois[78] using microtiter plates coated with biotin linked to albumin was extensively used. The assay as described by Mock[79] has the following sequence:

1. A constant amount of ^{125}I-avidin is incubated with known amounts of biotin and with dilutions of sample to produce the standard curve and unknown response.
2. An aliquot of the first incubation is transferred to microtiter plates coated with biotin covalently linked to bovine serum albumin.
3. The assay solutions are incubated in the coated wells for at least 4 h at room temperature.
4. The wells are washed with buffer and the bound ^{125}I is counted in a scintillation counter.

As biotin increases in the standard additions or unknown samples, more biotin-binding sites on the ^{125}I-avidin are occupied during the initial incubation. Thus, fewer radiolabels are bound to the well in the second incubation. Mock[79] fully describes the traditional methodology, and the reader is directed to his detailed procedural guide.

Later changes introduced biotinylated enzymes in place of the radiolabel. Use of radioligands are now unnecessary for biotin quantitation through the application of conventional enzyme-linked assays (ELAs). Biotinylated enzymes used in conjunction with avidin or strep-avidin include lysozyme,[84] glucose-6-phosphate dehydrogenase,[85] alkaline phosphatase,[86] pyrophosphatase,[87] and horseradish peroxidase (HRP).[51,88–92] As early as 1988, Finglas et al.[91] reported a procedure based on avidin-horseradish peroxidase conjugates and microtiter plate techniques for the assay of biotin in liver. This early use of EPBA was sensitive with a limit of detection of 10 pg biotin per well. Since avidin binds with biotin analogs, the EPBA was thought to compare closely with the *L. plantarum* assay.[80] The assay, however, has not been widely applied to foods.

Avidin-biotin binding assays have been developed using fluorescence labels and probes,[93–97] chemiluminescence,[98,99] and bioluminescence.[100–104] However, the avidin-HRP ELA has been the most widely accepted biotin assay. It provides a highly sensitive and specific off-line determinative step for LC assays as discussed in the following section. Competitive enzyme-linked immunosorbent assays (ELISA) using avidin- or streptavidin-conjugated HRP are also proven in the literature.[105–108]

12.3.3 Advances in analysis of biotin

12.3.3.1 Liquid chromatography

Application of LC was hampered owing to lack of sensitivity of ultraviolet (UV) and fluorescence detectors for native biotin and its metabolites. Use of derivatization reactions to form fluorescent derivatives and off-line, postcolumn HRP-avidin binding assays has provided the necessary specificity and sensitivity needed to make LC-based assays the approach of choice for accurate quantitation in foods and biologicals. LC procedures are summarized in Table 12.5[10,22,23,24, 108–131.] The methods are organized by detection system in Table 12.5.

Table 12.5 LC and LC-MS Methods for the Analysis of Biotin in Foods, Feeds, Pharmaceuticals, and Biologicals

Sample Matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Spectrophotometric detection					
Standards/biotin, biotin sulfoxide	—	Reversed-phase μBondapak C_{18}, 10 μm, 25 cm × 4.6 mm Mobile phase—isocratic 0.05% TFA:MeCN (50:50) Anion Exchange Aquapore Ax-300, 25 cm × 4.6 mm Mobile phase—gradient 0.05 M Tris—HCl, pH 4.5 to 6.5, 30 min	220 nm	—	*J. Chromatogr.*, 330, 153, 1985;[108] *Meth. Enzymol.*, 122, 63, 1986[109]
Multivitamins/multiminerals	Dissolve in 0.02 N NaOH, Florisil SEP-PAK	Zorbax ODS, 15 cm × 4.6 mm	200 nm	%Recovery—90	*Pharm. Res.*, 4, 261, 1987[110]
Almonds	Defat with HEX, sonicate at 75°C in H_2O/0.1 N HCl (1:7), adjust pH to 4.6 with 0.1 M NaOH, bring to volume, filter. Clean-up—Strong cation exchange SPE	Supelcosil LC-8-DB, 3 μm Mobile phase 5 mM hexane sulphonic acid containing 0.1% TEA:MeOH (80:15) Flow rate—1 mL min^{-1}	200 nm	%Recovery—79	*J. Chromatogr.*, 553, 187, 1991[111]
Pharmaceuticals	Sonicate in Hex, partition with phosphate buffer, filter	LC-18-DB, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic Phosphate buffer, pH 2.5/MeOH (85:15) Flow rate—1.5 mL min^{-1}	200 nm	%RSD—2.5	*J. Liq. Chromatogr. Rel. Technol.*, 26, 195, 2003[112]
Pharmaceuticals	Hanson SR8-Plus dissolution apparatus, 0.02 N phosphate buffer, pH 7.4	Supelcosil LC-18,5 μm, 2.5 cm × 4.6 mm Mobile phase—isocratic H_2O (pH 2.5):MeCN (80:20) Flow rate—1.5 mL min^{-1}	190 nm	DL—0.005 μg mL^{-1} QL—0.01 μg mL^{-1}	*J. Liq. Chromatogr. Rel. Technol.*, 27, 511, 2004[113]

Fluorescent Derivatives					
Standards/biotin, dethiobiotin, biotin sulfoxides, biotin sulfone	—	μBondapak C_{18}, 10 μm, 33 cm × 4.0 mm Mobile phase— THF:H_2O (50:50) or MeOH:water (60:40)	Precolumn derivatization w-4-dibromoacetophenone (BAP) or 4-bromomethyl methoxy coumarin (Br-Mmc) Ex λ = 360 Em λ = 410	DL—0.009–0.13 nmol	*Anal. Biochem.*, 128, 359, 1983[114]
Pharmaceuticals, serum	Extract with MeOH	Normal phase LiChrosorb Si60, 15 cm × 4 mm Mobile phase—isocratic HEX:IPA:H_2O (80:20:1) Flow rate—1.0 mL min^{-1} Reversed Phase— TSK—gel 80, 5 μm, 15 cm × 4 mm Mobile phase— H_2O:MeCN (43:57) Flow rate—1.0 mL min^{-1}	Pre–column derivatization 1-pyrenyldiazomethane Ex λ = 340, Em λ = 395	%Recovery—98.8	*J. Chromatogr.*, 456, 421, 1988[115]
Biologicals, gut tissue	Homogenize 2–3 g, in 5 mL of 5% TCA, add dethiobiotin as IS. Centrifuge, reextract 2×. Clean-up—SEP-Pak, C_{18} and Dowex 1 × 8 formate column. Retreat with SEP-Pak, C_{18}	Reversed-phase Hypersil ODS, 3 μm Mobile phase—isocratic H_2O:MeOH (60:40) Flow rate—1 mL min^{-1} Normal Phase Shandon Hypersyl, 3 μm Mobile phase—isocratic MeOH:CH_2Cl_2 (5:95) Flow rate—1.4 mL min^{-1}	Precolumn derivatization Panacyl bromide Ex λ = 380 Em λ = 470		*Anal. Biochem.*, 200, 89, 1992;[116] *Meth. Enzymol.*, 279, 286, 1997;[117] *Anal. Chem.*, 62, 2536, 1990;[118] *Anal. Chim. Acta*, 246, 103, 1991[119]
Pharmaceuticals, horse supplement/biotin , biocytin	Dilute solutions with mobile phase, extract feed supplement with 1.0 M NaOH, adjust pH to 6–7 with 1.0 HCl. Dilute with 0.1 M phosphate buffer, pH 6.0.	Reversed-phase Microsorb C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic 0.1 or 0.2 phosphate buffer (pH 6.0): MeOH (50:50) Flow rate—0.4 mL min^{-1}	Postcolumn derivatization Fluorescein isothiocyanate—avidin conjugate Ex λ = 490 Em λ = 520	DL (pg) Biotin—89 Biocytin—94	*J. Chromatogr. A*, 654, 79, 1993;[120] *Meth. Enzymol.*, 279, 275, 1997[121]

Continued

Table 12.5 (Continued)

Sample Matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Pharmaceuticals	Dissolve in H_2O, sonicate, centrifuge, filter	Tonsorb C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic 20 mM SDS (pH 3.5 with perchloric acid):MeCN (19:1) Flow rate—0.5 mL min^{-1}	Postcolumn derivatization *O*-phthaladehyde (OPA) and 3-mercaptopropionic acid (3-MPA) Ex λ = 342 Em λ = 453	%RSD—1.9–3.4 DL—10 ng	*J. Pharmceut. Biomed. Anal.*, 16, 1357, 1998[122]
Model system/biotin, biocytin		Microsorb C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic 0.1 M phosphate (pH 6.0):MeCN (50:50) Flow rate—0.4 mL min^{-1}	Precolumn competitive binding Avidin-(2-[4′-hydroxyphenylazo])-benzoic acid 345 nm	DL (mol L^{-1}) Biotin—7.3×10^{-6} Biocytin—9.7×10^{-6}	*Anal. Chem.*, 62, 2536, 1990[118]
Model system/biotin, biocytin, vitamin tablet		Microsorb C_{18}, 25 cm × 4.6 mm Mobile phase—isocratic 0.1 M phosphate (pH 6):MeOH (54:46) Flow rate—0.4 mL min^{-1}	Postcolumn competitive binding Avidin-2-anilino-naphthalene-6-sulfonic acid Fluorescence Ex λ = 328 Em λ = 438	DL (µg L^{-1}) Biotin—120 Biocytin—185	*Anal. Chim. Acta*, 246, 103, 1991[119]
Pharmaceuticals, foods/biotin, biocytin	To 5–10 g ground sample, add 1. 300 µL reduced flutathione, 300 µL EDTA, 30 mL citrat buffer, 3 mL papain. 2. Add 100 mg takadiastase if high starch sample. 3. Digest at 37°C overnight with shaking. 4. Cool, add H_2O to 50 mL, filter.	Reversed-phase Lichrosphere 100 RP, 5 µm, 25 cm × 5 mm, endcapped Mobile phase—isocratic 0.1 M phosphate (pH 6.0):MeOH (81:19) Flow rate—0.4 mL min^{-1}	Postcolumn derivatization Avidin-fluorescein Ex λ = 490 Em λ = 520	%Recovery—90–106 DL—0.005 µg g^{-1}	*Food Chem.*, 65, 253, 1999[22]

Body fluids, floods	Food Homogenize in water (1 part food/4 parts water) Add 1.5 mL of 3 mol L^{-1} HCl to 0.5 mL of the homogenate, incubate 2 h at 100°C. Cool, centrifuge, adjust pH to 2.5, filter.	Reversed-phase Sperisorb C_{18}, 25 cm × 2 mm Equilibrate column with 0.05% TFA (A), pH 2.5. Mobile phase—gradient 0.05% MeCN:0.05% TFA (1:1) (B) 0% B to 40% B over 35 min	Postcolumn HRP-avidin binding assay	$\%RSD_{Intra} \leq 5$ $\%RSD_{Inter}$—6	*J. Nutr.*, 131, 2208, 2001;[123] *J. Pediatr.*, 131, 456, 1997;[23] *J. Nutr.*, 129, 4945, 1999;[11] *Meth. Enzymol.*, 279, 265, 1997;[51] *J. Food Compos. Anal.*, 17, 767, 2004;[24] *Nutr. Biochem.*, 7, 518, 1996[124]
Dairy products, infant formula, medical nutritionals, premixes/biotin	Add 6% MPA, mix. SPE—SepPak C_{18} Elute with mobile phase.	Hypersil BDS C_{18}, 3 µm, 10 cm × 4 mm Mobile phase—isocratic MeOH:0.02 M phosphate (20:80), pH 7.0	Fluorescence Ex λ = 495 Em λ = 518 Postcolumn formation of conjugate with Streptavidin-fluorescein	Accuracy—98.8% of SRM 1846 Precision—3.48%	*J. AOAC Int.*, 89, 1515, 2006[128]
Electrochemical Detection					
Pharmaceuticals/biotin	To powdered tablet weight representing 50 µg mL^{-1} after dilution, add 30 mL of 0.05 M KH_2PO_4, sonicate, bring to 50 mL volume, filter	Reversed-phase LiChrosorb RP-18, 7 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:0.05 M KH_2PO_4, pH 2.6 (15:85) Flow rate—1.0 mL min^{-1}	+ 1.4 V vs Ag/AgCl (Reference)	DL—5–10 ng	*J. Chromatogr.*, 356, 326, 1986[125]
LC-MS					
Standards/biotin, dethiobiotin	—	Reversed-phase Hewlett-Packard RP-8, 5 µm, 10 cm × 3.2 mm Mobile phase—isocratic MeOH:water (40:60) Flow rate—0.6 mL min^{-1}	Formation of biotin methyl ester Mass spectrometer Positive ionization	—	*J. Chromatogr.*, 303, 272, 1984[126]
Skin	MeOH extraction	Reversed-phase Nucleosil 120-3 C_{18} Mobile phase—isocratic MeOH:water:HAC (90:9:1) Flow rate—0.2 µL min^{-1}	Mass spectrometer ESI, SIM mode	%RSD > 6.6 DL—800 pg mL^{-1} QL—5 ng mL^{-1}	*Anal. Comm.*, 34, 335, 1997[127]

Continued

Table 12.5 (Continued)

Sample Matrix/analyte	Extraction/clean up	Column/mobile phase	Detection	Quality assurance	References
Feed, food, tablets, premixes/ biotin	Alkaline extraction Add 5 g sample or one tablet into a 500 mL volumetric. Add 3 g $Na_2SO_3 \cdot 9H_2O$, 10 mL of 2% ammonium hydroxide and 250 mL H_2O, shake 20 min. Make to volume with water, filter, dilute to 0.01–1 μg mL^{-1}. To 1 mL filtrate add d_6-biotin (IS). Sulfuric acid extraction To 10 g sample add 100 mL 2 N H_2SO_4 and 1 mL d_6-biotin (4 μg mL^{-1}, IS). Autoclave, 30 min. Adjust to pH 5.7 with NaOH. Dilute with water Papain digestion Apply after H_2SO_4 extraction. After adjusting to pH 5.7, add glutathione, NaEDTA, citrate buffer and papain solution. Digest at 37°C for 16 h	System A for concentrations of 0.01–1 μg mL^{-1} Symmetry Shield C_{18}, 3.5 μm, 5 cm × 2.1 mm Mobile phase—gradient A—water with 0.1% formic acid B—MeOH with 0.1% formic 0–2.5 min—5% B, 2.5–7.0 min—to 75% B, 7.0–9.9 min—75% B, 9.9–10 min—to 5% B Flow rate—0.25 mL min^{-1} System B for concentrations of 0.5–50 ng mL^{-1} Column-switching System A w/ heart-cut switching to a Vydac 218TP5215, 5 μm, 15 cm × 2 mm Mobile phase—gradient	LC-MS/MS-ESI Positive ion mode	%RSD—2.3–9.2	*J. Chromatogr. B*, 831, 8, 2006[129]

Beverage/biotin	Dilute with water to 4–20 µg mL^{-1}. SPE—Sep-Pak Plus + C18ENV. Elute with MeOH.	L-column, ODS, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic 20 mM KH_2PO_4:MeCN (75:25) Flow rate—1 mL min^{-1}	Precolumn derivatization with 2-nitrophenylhydrazine using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl as the coupling reagent LC-MS-ESI Positive ion-mode or negative ion-mode	%Recovery—97.5 (LC-MS)	*J. Chromatogr. A*, 1142, 231, 2007[130]
LC-ELSD and LC-MS					
Multivitamin, multielement tablets (NIST SRM 3280)/biotin	LC-ELSD Spike with desthiobiotin (IS). Dilute with 1.5% formic acid. Shake and centrifuge. Supernatant is treated with SPE (C_{18}) to extract biotin, elute with 1.5% formic acid in water:EtOH (50:50). LC-MS Spike with [$^2H_2$]-biotin (IS). Dilute with MeOH. Mix, heat to 60°C, sonicate. Centrifuge, filter 1 mL through cellulose filter (0.45 µm).	LC-ELSD Supelcosil LC-CN cyanopropyl, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic Solvent A—1% formic acid in water Solvent B—0.1% formic acid in MeOH (A:B—95:5) Flow rate—1 mL min^{-1} LC-MS Waters xTerra RP C_{18}, 5 µm, 15 cm × 4.6 mm Mobile phase—isocratic Solvent A—0.1% formic acid in water Solvent B—0.1% formic acid in MeOH (A:B—77:23) Flow rate—350 µL min^{-1}	LC-ELSD SEDEX 75 detector LC-MS-ESI Positive-ion mode	%RSD 1.1 (LC-MS) 6.7 (LC-ELSD) DL (ng) 0.02 (LC-MS) 155 (LC-ELSD) QL (ng) 0.06 (LC-MS) 310 (LC-ELSD)	*J. Agric. Food Chem.*, 54, 8710, 2006[131]

12.3.3.1.1 Extraction procedures for analysis of biotin by LC. For analysis of high concentration pharmaceuticals, simple extraction with base,[110,121] buffer,[112,113,124] or methanol[115] can suffice. Some investigators have followed liquid extractions with SPE[110,111,116,117] cleanup procedures. Extractions with acid have commonly been used before analysis of foods to ensure liberation of bound biotin forms. Staggs et al.[24] used acid hydrolysis with HCl before LC assay with the HRP-avidin binding assay as the determinative step. Lahély et al.[22] digested various foods with papain and takadiastase, preferring the enzymatic hydrolysis over sulfuric acid hydrolysis. These authors showed that autoclaving of beef liver for 3 h in 3 M sulfuric acid was necessary to free biotin and led to erratic results. The enzyme digestion did not induce degradation.

12.3.3.1.2 Chromatography parameters

Supports and Mobile Phases

Almost all published LC methods use reversed-phase with C_{18} supports. Chastain et al.[108] showed that reversed-phase chromatography on C_{18} was superior to anion-exchange LC for the resolution of biotin and 13 metabolites and analogs of biotin. Although anion-exchange separations gave shorter run times, fewer analogs were resolved. Chastain's procedure formed the basis of more recent LC applications for biotin research. In the original method,[108] a 35 min linear gradient from 0.05% trifluoroacetic acid, pH 2.5 to 0.05% trifluoroacetic acid: acetonitrile (70:30) successfully resolved the standards. Spectrophotometric detection at 220 nm limited the application of the method. Mock's research group[10,23,24,51,123] modified the original mobile phase and gradient elution to include equilibration of the C_{18} support with 0.05% trifluoroacetic acid buffered with ammonium acetate, pH 2.5 (A) and forming a linear gradient with 0.05% trifluoroacetic acid:acetonitrile (1:1) (B). The gradient runs for 35 min, reaching 40% B. This method coupled to an off-line HRP-avidin binding assay yields a detection limit of 5 fmol.[51]

Detection of Biotin

Although usable for pharmaceuticals, UV detection is not suitable for foods or other biologicals. Only weak absorbance exists at 200–220 nm with an absorption maxima at 204 nm.[38] Various fluorescent derivatives have been formed precolumn[114–117] or postcolumn[22,119–122] to increase sensitivity to allow assay of biological levels of biotin and its metabolites. Desbene et al.,[114] in early application of fluorescence, produced *p*-bromophenacyl bromide esters and 4-bromomethylmethoxy-coumarin derivatives for UV and fluorescence detection, respectively. Later, researchers used fluorescent labeling with 1-pyrenyldiazomethane,[115] panacyl bromide,[116,117] fluorescein isothiocyanate-avidin conjugate,[22,120,121,128] and avidin-2-anilinonaphthalene-6-sulfonic acid.[119]

Stein et al.[116] developed an LC method useful for complex matrices on the basis of the formation of fluorescent esters of biotin with panacyl bromide in the presence of crown ether. The esters are formed precolumn and resolved either by normal- or reversed-phase chromatography (Figure 12.3). A disadvantage of the method compared to avidin-horseradish peroxidase/LC method is the extensive cleanup required of biological sample extracts before derivatization. Nevertheless, the method shows the advances being made in analytical method development for biotin, its metabolites, and analogs.

A postcolumn reaction with avidin labeled with fluorescein isothiocyanate (FITC) was developed by Przyjazny et al.[119] for the quantitation of biotin and biocytin. The procedural details of the avidin-FITC method and a method using the fluorescent probe 2-anilinonaphthalene-6-sulfonic acid for postcolumn reaction with biotin and biocytin were given by Hentz and Bachas[121] in *Methods of Enzymology*, vol. 279. In-depth discussions on the above procedures and other approaches for biotin assay are provided in this source.

The power of LC resolution has been put into a practical and more routine working situation for biotin assay by the successful coupling by the LC method of Chastain et al.[108] with

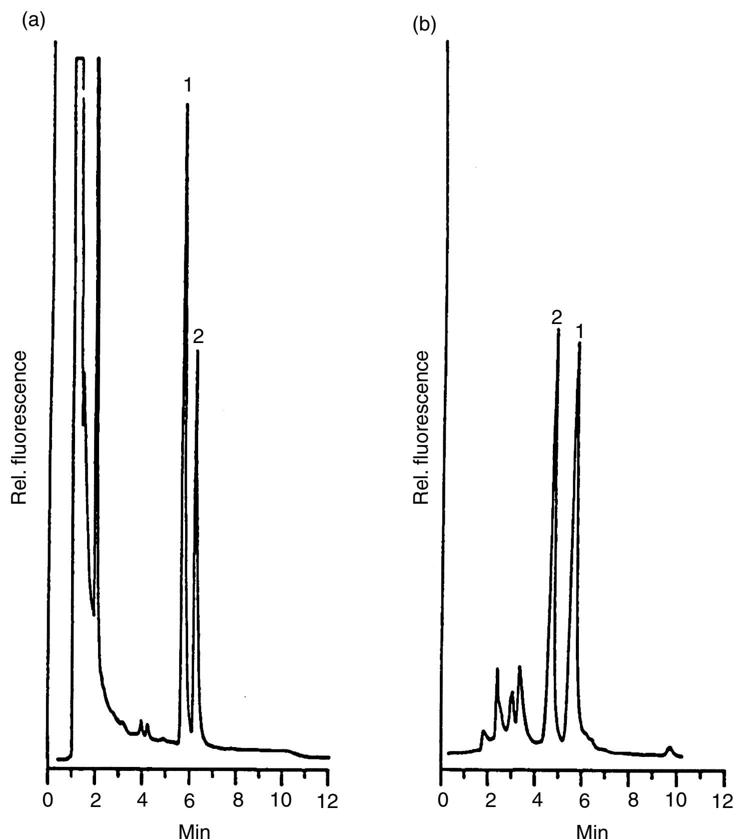

Figure 12.3 Resolution of panacyl esters of dethiobiotin (1) and biotin (2). (a) Normal-phase, (b) Reversed-phase. (Reproduced from Stein, J., Hahn, A., Lembcke, B., and Rehner, G., *Anal. Biochem.*, 200, 89, 1992. With permission.)

off-line avidin-binding detection using avidin-HRP. Research applications of the method include papers by Zemplenie and Mock,[10] Zempleni et al.[124] and Mock et al.[23] that demonstrate the excellent specificity and sensitivity of the procedure. The research conclusively shows that without resolution by LC, a direct assay of total avidin-binding substances in serum or urine overestimates biotin and underestimates the total biotin plus biotin metabolites.[10,51,123,124] The analysis applied to foods[24] has been shown to greatly improve analytical data for biotin content of food. Likewise, older data for biotin content is quite suspect.

Procedural details of the LC-HRP-avidin assay are provided by Mock.[51] Steps of the assay are summarized by Mock[5] as follows:

1. Biotin and biotin metabolites are resolved by LC.
2. *Incubation 1*: HRP-avidin is incubated with LC fractions containing biotin or its metabolites.
3. The biotin or biotin metabolite binds to HRP-avidin, occupying some of the total biotin-biding sites.
4. *Incubation 2*: An aliquot of the mixture is transferred to a well previously coated with biotinyl-bovine serum albumin (BSA). HRP-avidin molecules with unoccupied biotin-binding sites will bind to the biotinyl-BSA.
5. After Incubation 2, free HRP-avidin is washed away.
6. *Incubation 3*: The amount of HRP-avidin bound to the microplate well is quantitated by measuring the rate of peroxidation of *o*-phenylenediamine (OPD).

In 2006 and 2007, three methods were published using LC combined with mass spectrometry (LC-MS) that have the potential to improve biotin assay.[129–131] Höller et al.[129] provided extensive information on LC-MS/MS assay of a wide variety of matrices using deuterated biotin (d_6-biotin) as the internal standard. Electrospray ionization (ESI) with positive ionization was shown to be the most sensitive ionization method. Höller et al.[129] details alkaline, sulfuric acid, and pepsin digestion procedures for use with feed, foods, tablets, and premixes. Yomoto and Ohnishi[130] used precolumn derivatization and MS-ESI to assay biotin as 2-nitrophenylhydrazine derivatives. The LC detection limit was 0.6 ng at 400 nm and 0.025 ng with selected ion monitoring of the $[M + H]^+$ ion of the derivatives.

Nelson et al.[131] developed an LC-evaporative light-scattering detection (ELSD) method with desthiobiotin as the internal standard and an LC-MS method using deuterated $[^2H_2]$-biotin as the internal standard. These methods were developed to improve quantification of biotin in NIST Standard Reference Material 3280:Multivitamin/Multielement Tablets. Both methods were deemed applicable to the analysis; however, the %RSD for the LC-MS method was 1.1 compared to 6.7 for the LC-ELSD method. The limit of detection and limit of quantification were 0.02 ng and 0.06 ng, respectively, by LC-MS and 1.55 ng and 310 ng, respectively, by LC-ELSD.

As with other water-soluble vitamins, LC-MS methods are advantageous to other well-founded biotin analytical methods. Over the past 15 years, methods have dramatically improved, and LC-MS will greatly add to the abilities to quantify biotin.

12.3.3.2 Optical biosensor-based immunoassays

The Biacore AB company has successfully introduced immunoaffinity-based optical biosensor assays for several water-soluble vitamins, including biotin.[132–135] The automated assay utilizes analyte detection through coupling of the antibody–antigen interaction through optical or electrochemical signal transduction. The antibody or antigen is immobilized on the transducer surface of the immunosensor with a gold support layer. The principle of the biosensor assay uses surface plasmon resonance optics enabling the continuous, nonlabeled monitoring of a sensor-bound, ligand-analyte interaction. The Biacore AB system achieves the requirements of the biosensor through a flexible hydrophilic carboxymethyldextran polymer linked to an alkythiol-modified gold surface.

Assay of biotin in infant formula and milk by the Biacore AB system gave excellent performance characteristics.[132] Assay of NIST 1846 “Infant Formula” Standard Reference Material gave a between-laboratory RSD_r of 0.9%. Overall recoveries ranged from 86% to 102% with an extremely low quantitation range of 2–70 ng mL^{-1}. Biotin values obtained for commercial products matched those obtained by microbiological assay. The study shows that the optical biosensor assay provides a simple and fast answer to the quantification of biotin. The method was recently granted PMT Status (Performance Tested Methods) by the AOAC Research Institute.[135] Complete validation parameters for the Biacore Qflex Biotin Kit are provided in the publication.

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chapter thirteen

Pantothenic acid

13.1 Review

Pantothenic acid was fractionated from bios (Chapter 12, Section 12.2) as an acid component in 1931. It was identified and named by R. J. Williams in 1933. The name was derived from the Greek word "panthos," meaning everywhere, which indicates its widespread occurrence in nature. Williams et al.[1] determined the structure in 1939. Other significant historical events include relating the chick antidermatitis factor to pantothenic acid (1939),[2,3] synthesis (1940),[4] and identification of the structural role in coenzyme A (CoA).[5] A Nobel Prize was awarded to Lipmann and Krebs in 1953 for their research on the function of CoA in metabolism. Recognition of the acyl-carrier protein (ACP) and its role in acyl group transfer (fatty acid synthesis) occurred in 1965.[6]

Because of the availability of pantothenic acid in the food supply, human deficiency has been diagnosed only in severely malnourished patients.[7,8] The abundance in nature results, in part, from its structural role in CoA. From 1930 to 1950, various animal species were studied under conditions that induced pantothenic acid deficiency.[9–17] The deficiency in humans was difficult to characterize because of inconsistencies in chemical and clinical symptoms induced by diets devoid of pantothenic acid or the use of pantothenic acid antagonists.[18–24] Inducement of the deficiency in humans produced variable symptoms indicative of other vitamin deficiencies or possible interrelationships with other nutritional deficiencies. Because of the lack of a clear biological marker, human status indicators are difficult to interpret. General symptoms, common to many nutritional deficiencies noted in the earlier cited studies on humans included irritability and restlessness, fatigue, apathy, malaise, sleep disturbances, nausea, vomiting, abdominal cramps, neurological symptoms (numbness, abnormal sensations in the hands and feet, muscle cramps, staggering gait), hyperglycemia, and increased sensitivity to insulin.[25]

Intake levels used by the Dietary Reference Intake (DRI) committee in setting the adequate intake (AI) (Table 13.1) for adults were largely based on a 1981 study[26] that indicated pantothenic acid intakes from food averaged 2.9 mg 1000 $kcal^{-1}$. Clinical measures include whole blood levels, which usually range from 1.57 to 2.66 μmol L^{-1}. Sauberlich and Skala[27] had reported that blood levels below 100 mg 100 mL^{-1} may indicate low dietary intake. Total pantothenic acid in urine, plasma, and erythrocytes have been used to indicate status; however, their sensitivity and interpretation is problematic.[28] Whole blood and erythrocyte concentrations are highly correlated; but plasma levels are weakly correlated to intake and status.[25]

Most foods contain measurable pantothenic acid levels because of its diverse metabolic functions as a structural component of CoA. Richest food sources are yeast, organ meats,

Table 13.1 Dietary Reference Intakes for Pantothenic Acid

Life stage	DRI (mg d^{-1})
Infants (months)	
0–6	1.7
7–12	1.8
Children (years)	
1–3	2
4–8	3
Males (years)	
9–13	4
14–18	5
19–30	5
31–50	5
51–70	5
>70	5
Females (years)	
9–13	4
14–18	5
19–30	5
31–50	5
51–70	5
>70	5
Pregnancy (years)	
≤18	6
19–30	6
31–50	6
Lactation (years)	
≤18	7
19–30	7
31–50	7

Bold type: Recommended Dietary Allowance: ordinary type: adequate intake (AI)

Source: Food and Nutrition Board, Institute of Medicine, *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B-6, Folate, Pantothenic Acid, Biotin and Choline*, National Academy of Sciences Press, Washington, DC, 2000, chap. 10.

egg yolk, and whole grains. Relatively low amounts are present in fresh and cooked vegetables, nuts, fruits, meat, milk, and processed cereals.[29] However, these foods are consumed in quantity in most diets and represent significant dietary sources. Table 13.2 gives food values from the United States Department of Agriculture (USDA) National Nutrient Database for Standard Reference, Release 19.[30] As with other vitamins supplemented into breakfast cereals, such products are the most concentrated food sources for pantothenic acid. Organ meats, mushrooms, chicken, and cereal grains represent quite concentrated sources. Most vegetables contain less than 1 mg 100 g^{-1}. Daily intakes in the United States range from 4 to 7 mg.[25] Because of lack of availability of complete food composition data, most nutrient databases available internationally do not include pantothenic acid. The USDA has attempted to rectify this situation by including food composition information when reliable sources become available. The Dietary Reference Intake committee did not set tolerable upper intake levels (ULs), since no reports exist of adverse effects of high intake.[25] The Reference Daily Intake (RDI) set by the Food and Drug Administration is 10 mg.[29]

Table 13.2 Pantothenic Acid Content of Various Foods[a]

Description	NDB no.	mg 100 g^{-1}	Description	NDB no.	mg 100 g^{-1}
Cereals ready-to-eat, GENERAL MILLS, Whole Grain TOTAL	08077	35.5	Sweet potato, cooked, baked in skin, without salt	11508	0.9
Cereals ready-to-eat, KELLOGG, KELLOGG'S Complete Wheat Bran Flakes	08028	34.9	Peas, edible-podded, frozen, cooked, boiled, drained, without salt	11303	0.9
Cereals ready-to-eat, KELLOGG, KELLOGG'S PRODUCT 19	08058	33.6	Mushrooms, canned, drained solids	11264	0.8
Cereals ready-to-eat, GENERAL MILLS, TOTAL Corn Flakes	08246	33.0	Milk, canned, condensed, sweetened	01095	0.8
Cereals ready-to-eat, GENERAL MILLS, TOTAL Raisin Bran	08247	18.0	Milk, canned, evaporated, nonfat	01097	0.7
Seeds, sunflower seed kernels, dry roasted, with salt added	12537	7.1	Turkey, all classes, neck, meat only, cooked, simmered	05180	0.7
Beef, variety meats and by-products, liver, cooked, pan-fried	13327	6.9	Fish, salmon, sockeye, cooked, dry heat	15086	0.7
Chicken, liver, all classes, cooked, simmered	05028	6.7	Peas, edible-podded, cooked, boiled, drained, without salt	11301	0.7
Mushrooms, shiitake, cooked, without salt	11269	3.6	Corn, sweet, yellow, canned, vacuum pack, regular pack	11176	0.7
Braunschweiger (a liver sausage), pork	07014	3.4	Lentils, mature seeds, cooked, boiled, without salt	16070	0.6
Turkey, all classes, giblets, cooked, simmered, some giblet fat	05172	2.5	Yogurt, plain, skim milk, 13 g protein per 8 ounce	01118	0.6
Mushrooms, cooked, boiled, drained, without salt	11261	2.2	Broccoli, cooked, boiled, drained, without salt	11091	0.6
Mushrooms, raw	11260	1.5	Peas, split, mature seeds, cooked, boiled, without salt	16086	0.6
Oat bran, raw	20033	1.5	Parsnips, cooked, boiled, drained, without salt	11299	0.6
Fish, trout, rainbow, farmed, cooked, dry heat	15241	1.3	Dates, deglet noor	09087	0.6
Chicken, broilers or fryers, dark meat, meat only, cooked, fried	05044	1.3	Yogurt, plain, low fat, 12 g protein per 8 ounce	01117	0.6
Couscous, dry	20028	1.2	Sweet potato, cooked, boiled, without skin	11510	0.6
Rice, white, long-grain, par-boiled, enriched, dry	20046	1.1	Orange juice, frozen concentrate, unsweetened, undiluted	09214	0.6
Bulgur, dry	20012	1.0	Sweet potato, canned, vacuum pack	11512	0.5
Rice, white, long-grain, regular, raw, enriched	20044	1.0	Potatoes, mashed, home-prepared, whole milk and margarine added	11371	0.5
Wheat flour, whole-grain	20080	1.0	Potatoes, mashed, home-prepared, whole milk added	11657	0.5
Chicken, broilers or fryers, breast, meat and skin, cooked, fried, flour	05059	1.0	Tomato products, canned, puree, without salt added	11547	0.4
Turkey, all classes, meat only, cooked, roasted	05168	0.9	Pumpkin, canned, without salt	11424	0.4
Potatoes, hashed brown, home-prepared	11370	0.9	Beans, baked, canned, with pork and tomato sauce	16011	0.4

[a] Data from USDA National Nutrient Database for Standard Reference, Release 19

Pantothenic acid occurs in the free state, as pantethine, bound to proteins, or in CoA. In the acyl carrier protein (ACP), 4′-phosphopantotheine is the prosthetic group that provides the binding site for two-carbon fragments. CoA is the acyl carrier for oxidative removal of acyl groups, and ACP is the carrier of acyl groups for synthesis of fatty acids. Characteristic reactions requiring CoA are regulation of alcohols, amines, and amino acids, and the oxidation of pyruvate by the pyruvate dehydrogenase complex to form acetyl-CoA. β-Oxidation of fatty acids results in the removal of two-carbon fragments transferred as acetyl-CoA. CoA participates in condensation reactions to form a variety of intermediary metabolites including formation of citric acid from oxaloacetic acid in the tricarboxylic acid (TCA) cycle. Many proteins have been identified that are modified through acetylation by CoA. Metabolic roles of such modified proteins in human metabolism are discussed by Plesofsky.[31] Specific proteins that are known to be significant to signal transduction include the Ras proteins, membrane receptors, and protein kinases.

13.2 Properties

13.2.1 Chemistry

13.2.1.1 General properties

The structure of pantothenic acid is given in Figure 13.1. The compound is systematically named d(+)-*N*-(2,4-dihydroxy-3,3-dimethyl-l-oxobutyl)-β-alanine.[32] The trivial name is d(+)-α,γ-dihydroxy-β,β-dimethylbutryl-β-alanine. It consists of pantoic acid linked through an amide linkage to β-alanine. The name, pantoyl-β-alanine, should not be used. Owing to the chirality at the hydroxylated carbon atom of the pantoic acid moiety, the vitamin is optically active and a racemic mixture results from synthesis. Only the d(+)-enantiomers are biologically active and present in nature. The structure of coenzyme A is provided in Figure 13.2. Biosynthesis of coenzyme A occurs through the following steps:[24,31]

1. Pantothenic acid is phosphorylated to pantothenic acid 4′-phosphate.
2. Pantothenic acid 4′-phosphate condenses with cysteine to yield 4′-phosphopantothenoyl-cysteine.
3. The 4′-phosphopantothenoyl-cysteine is decarboxylated to 4′-phosphopantetheine (Figure 13.2).
4. Andenosine 5′-monophosphate is added with phosphorylation of the ribose 3′-hydroxyl to produce the final structure of CoA.

The cysteine sulfhydryl group (–SH) is the active site for esterification with acetate or acyl groups. Also, 4′-phosphopantetheine may be covalently bound to proteins such as the ACP[31] (Figure 13.2).

The United States Pharmacopeial Convention (USP) standard is d(+)-pantothenic acid. Commercial forms include the sodium and calcium salts and the alcohol, pantothenol.

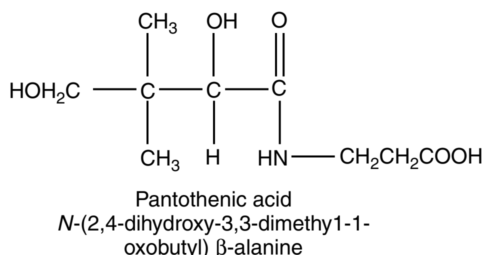

Figure 13.1 Structure of pantothenic acid.

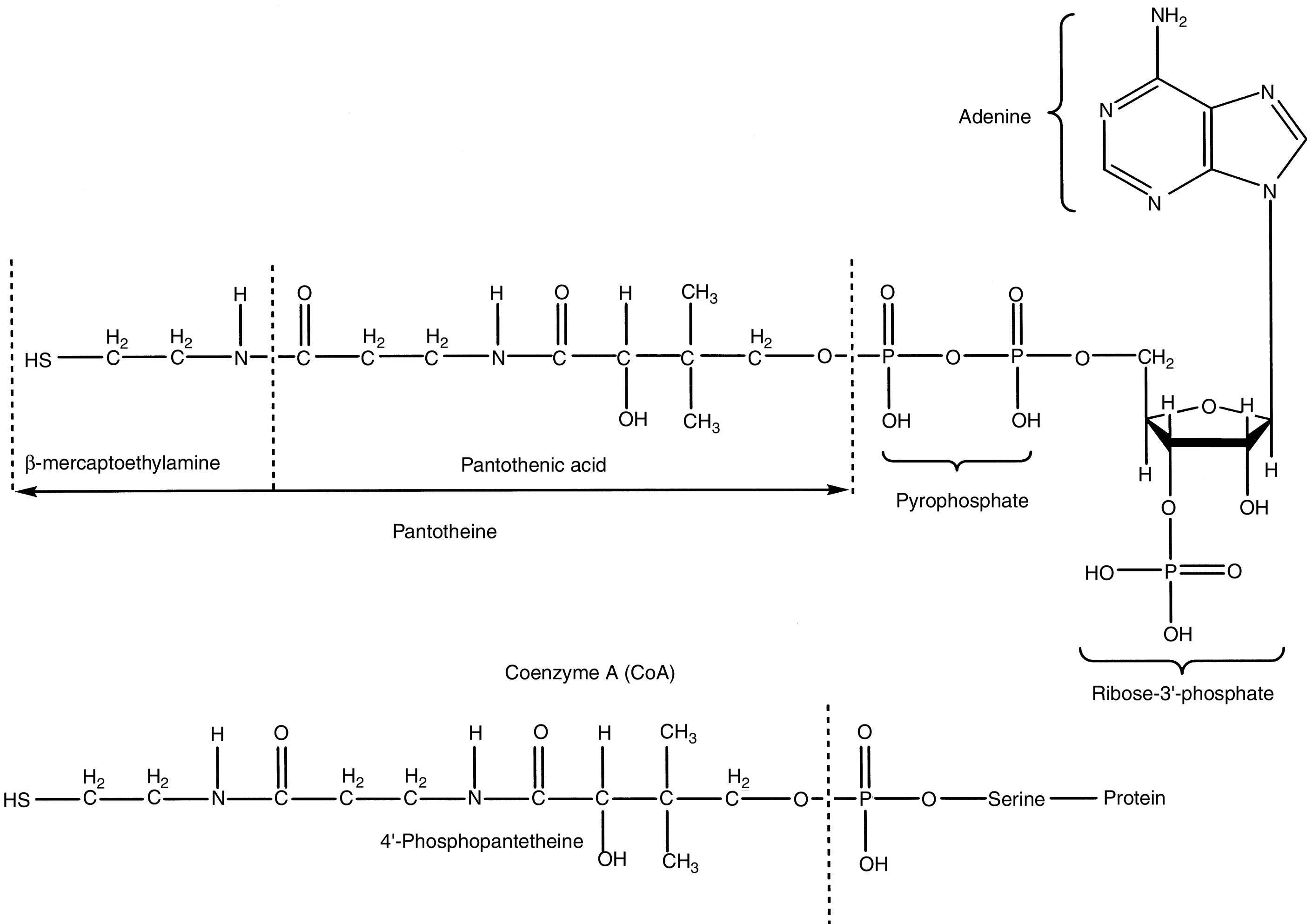

Figure 13.2 Structure of coenzyme A.

Pantothenic acid is highly hygroscopic and more unstable than the salts. Therefore, the salts, predominantly calcium pantothenate, are the usual forms chosen for food fortification and dry pharmaceutical products. Pantothenol is a hygroscopic viscous oil. It is commonly used in liquid pharmaceuticals. The alcohol is slightly soluble in water and very soluble in alcohol.[33] Pantothenol is equivalent to pantothenic acid in biological activity. The alcohol has better stability at pH 3–5 compared to the salts.[29] Equivalent to 1 g of calcium pantothenate is 0.92 g of pantothenic acid, and 1 g of pantothenol is equivalent to 1.16 g of calcium pantothenate.[34] Physical properties are summarized in Table 13.3.

13.2.1.2 Spectral properties

Pantothenic acid and closely related compounds do not contain a chromophore. The carbonyl group weakly absorbs below 210 nm.

13.2.2 Stability

In solution, pantothenic acid is most stable at pH 4.0–5.0.[33] For food fortification and pharmaceutical uses, calcium pantothenate is usually used because of greater stability under most environmental conditions, particularly near neutrality.[33] Pantothenic acid will hydrolyze to pantoic acid and β-alanine under both basic and acidic conditions.[35]

Large losses can occur through leaching into the cooking water during preparation of vegetables and through heat processing. Schroeder,[37] reporting on a large study completed at USDA and originally compiled in USDA Agriculture Report 36[38] involving 723 foods, found that pantothenic acid losses due to canning ranged from 46% to 78% in vegetables. Losses in meats were lower owing to canning (20%–35%). In frozen foods, the pantothenic

Table 13.3 Physical Properties of Pantothenic Acid, Salts, and Pantothenol

Substance[a]	Molar mass	Formula	Solubility	Melting point °C	Crystal form	λ max nm
Pantothenic acid CAS No. 79-83-4 **7147**	219.24	$C_9H_{17}NO_5$	Freely soluble in water, ethyl acetate, dioxane Moderately soluble in ether, amyl alcohol		None, oil	204 Very weak
Sodium pantothenate CAS No. 867-81-2 **7147**	241.20	$C_9H_{16}NNaO_5$	Soluble in water Sparingly soluble in ether, amyl alcohol Insoluble in $CHCl_3$	122–124	Crystals	No chromophore
Calcium pantothenate CAS No. 137-08-6 **7147**	476.53	$C_{18}H_{32}CaN_2O_{10}$	Soluble in water Slightly soluble in alcohol and acetone	195–196 (Dec.)	Minute needles	No chromophore
D(+) Pantothenol CAS No. 81-13-0 **2988**	205.25	$C_9H_{19}NO_4$	Freely soluble in alcohol, methanol Slightly soluble in water, ether		None, viscous liquid	No chromophore

[a] Common or generic name; CAS No: Chemical Abstract Service number, bold print designates the Merck Index monograph number.

Source: Ball, G.F.M., Chemical and biological nature of the water-soluble vitamins, In *Water-Soluble Vitamin Assays in Human Nutrition*, Chapman and Hall, New York, 1994, chap. 2; Budavari, S., *The Merck Index*, 13th ed., Merck and Company, Whitehouse Station, NJ, 2001, pp. 7087, 7082.3.

acid losses ranged up to 57% in vegetables and 70% in meats. Cheng and Eitenmiller[39] determined total pantothenic acid in spinach and broccoli at various processing points, comparing water to steam blanching. For both vegetables, steam blanching caused lower losses compared to water blanching. Steam blanching of spinach resulted in retention of 87% of the pantothenic acid, while retention after water blanching was 36%. Similar trends were apparent for broccoli, although losses were less than those noted for spinach. Canning of spinach caused a further significant loss after the blanching treatments. Earlier, Orr and Watt[40] reported that canned spinach retained only 29% of the pantothenic acid content of the raw product. Wituszynaska[41] also reported that retorting of combination fish and vegetable products resulted in 60% less pantothenic acid in the canned product compared to the fresh material from which they were made.

Stability in legumes during cooking shows that presoaking the beans and peas produces variable degrees of retention.[42,43] Pantothenic acid is higher in steamed legumes compared to boiled products. Early review articles are available that give insight into general processing effects on nutritional value that include pantothenic acid.[44–51] More recent data on the pantothenic acid content of processed foods determined by radioimmunoassay (RIA) were reported by Walsh et al.[52] They examined 75 ready-to-eat foods for pantothenic acid content. These authors found a relatively low amount of pantothenic acid in many highly processed foods. In milk, pantothenic acid is stable to pasteurization, since the normal pH of milk is in the optimal pH stability range. Goldsmith et al.[53] showed that pantothenic acid in human milk withstood a wide range of pasteurization conditions including low temperature long time (LTLT) at 62.5°C for 30 min, high temperature short time (HTST) at 72°C for 15 s and 88°C for 5 s, and sterilization at 100°C for 5 min. Only biotin, niacin, and riboflavin were as stable as pantothenic acid among the nine water-soluble vitamins studied. Further, pantothenic acid was not affected by frozen storage at 220°C for 1 month.

Pantothenase, a bacterial amidase, cleaves pantothenic acid into β-alanine and pantoic acid.[54] Action of this enzyme provides an enzymatic route for pantothenic acid destruction and, possibly, can play a role in stability in raw, unprocessed foods. Unlike many vitamins, pantothenic acid is stable to oxidative environments and light exposure.

13.2.3 Bioavailability

The first definitive information on the bioavailability of pantothenic acid from the American diet was provided from the original studies by Tarr et al. [55] Urinary excretion of pantothenic acid was assayed microbiologically in order to assess the bioavailability. Bioavailability ranged from 40% to 61% with a mean of 50%. The excretion of pantothenic acid was dose dependent with a parabolic increase in excretion rate occurring between 0 and 10 mg day^{-1} intake. Early research on pantothenic acid showed that CoA and phosphopantetheine contained most of the pantothenic acid occurring naturally in food.[56–59] Novelli et al.[60] showed that bound forms must be hydrolyzed to liberate panothenic acid before microbiological assay. It is now known that bioavailability depends upon hydrolysis of CoA and phosphopantetheine to free pantothenic acid by pantetheinase in the intestinal lumen, before absorption in the jejunum.[61]

Other than the research by Tarr et al.,[55] little definitive data exists on the bioavailability of pantothenic acid. Studies on wet and dry milled corn bran indicated that the bioavailability of pantothenic acid, niacin, and thiamin was somewhat dependent upon the particle size of the bran, indicating less enzymatic liberation of the vitamins from the coarser bran particles.[62] The degree to which particle size affected bioavailability was greater in wet milled corn bran than in dry milled bran. Bioavailability of pantothenic acid from wheat, potatoes, and boiled pork and beef meals measured in pigs with end-to-end ileo-rectal anastomosis permitting digesta to pass straight from the ileum to the rectum indicated pantothenic acid bioavailability of

65%–81%. Differences were not significantly different among the various diets but bioavailability values decreased in the order wheat diet > pork diet > potato diet > beef diet.[63]

13.3 Methods

Methods for assay of pantothenic acid vary widely in approach. However, liquid chromatography (LC) methods to analyze biological samples were slow to develop because of the lack of sensitive and specific detection modes. Thus, applications of LC were limited to the analysis of pharmaceuticals, vitamin premixes, and special dietary foods such as infant formula, in which the vitamin is present in higher concentration compared to nonformulated foods. Historically, methodology for pantothenic acid assay relied on various chemical and physical methods, animal bioassays, microbiological methods, gas chromatographic, and immunological methods. Older literature sources discuss the animal assays, microbiological, and chemical and physical methods in detail. These sources include Strohecker and Henning,[64] Baker and Frank,[65] and Freed.[66] Chemical and physical methods are available for the determination of pantothenic acid and its salts in dry or liquid vitamin preparations. Such procedures use hydrolysis under acidic or basic conditions, or reductive splitting of the vitamin into end products (β-alanine, pantoic acid, pentoyl lactone) that can be measured by spectrophotometry, fluorometry, or gas chromatography (GC). Reviews written by Wyse et al.,[67] El-Habashy and Eitenmiller,[29] Eitenmiller and Landen,[68] Ball,[35] and Velisek and Davidek[33] provide much method background information. More recently, LC, LC combined with mass spectrometry (LC-MS), and GC-MS methods have been developed for food analysis. Regulatory and handbook methods provided in Table 13.4 are mostly limited to microbiological and titration procedures for assay of vitamin tablets and pantothenol preparations. The USP/NSF[69] methods include an LC procedure for pharmaceutical preparations with detection at 210 nm. Association of Official Analytical Chemists (AOAC) International[70] methods are microbiological assays by *Lactobacillus plantarum* ATCC 8014. Method 945.74 (45.2.05) "Pantothenic Acid in Vitamin Preparations" does not include an enzymatic hydrolysis treatment and is limited to assay of free pantothenic acid and its salts. Method 992.07 (50.1.22) "Pantothenic Acid in Milk-Based Infant Formula" includes the traditional enzyme treatments (Section 12.3.3.1) for natural products, but has not been collaborated for other food matrices. AOAC International[70] does not provide LC-based methods.

13.3.1 Microbiological assay of pantothenic acid

L. plantarum ATCC 8014 has been the preferred microorganism for pantothenic acid assay for decades. Other organisms, both bacteria and protozoa, are suitable for the assay (*Saccharomyces cerevisiae*, *Saccharomyces uvarum*, *Lactobacillus delbrueckii*, and *Tetrahymena pyriformis*).[29] *L. plantarum* responds on an equimolar basis to free pantothenic acid and pantetheine. It does not respond to phosphopantetheine or intact forms of CoA.[75] Voigt et al.[76,77] completed in-depth studies of factors that interfere with microbiological assays and reported that *L. plantarum* was affected to a lesser degree than other commonly used organisms by sample matrix effects.

Because of the requirement of *L. plantarum* for free pantothenic acid and pantetheine, biological samples must be treated enzymatically by a "double-enzyme" treatment with alkaline phosphatase and pantetheinase that cleaves pantothenic acid from CoA and dephosphorylates pantetheine. Pantetheinase preparations from hog kidney, chicken liver, or pigeon liver are normally used. Endogenous pantothenic acid must be removed from enzyme preparations before the addition of sample digests. These procedures are provided in detail by Wyse et al.[67] Sample preparation procedures for the microbiological and radioimmunoassays (Section 13.3.2) are also provided in this excellent, highly descriptive

Table 13.4 Regulatory and Handbook Methods for the Analysis of Pantothenic Acid

Source	Form	Methods and application	Approach	Most current cross-reference
***U.S. Pharmacopeia, National Formulary,* USP 29/NF 24 Dietary Supplements Official Monographs, 2006**[69]				
1. Pages 2392, 2394–2396, 2398–2399, 2403, 2417–2419	Pantothenic acid as calcium pantothenate	Pantothenic acid in oil- and water-soluble vitamin capsules/tablets, oral solution w/wo mineral	Method 1—HPLC 210 nm Method 3—HPLC 205 nm Method 2—Microbiological	None
2. Pages 2428, 2430, 2432, 2434, 2436	Pantothenic acid as calcium pantothenate	Pantothenic acid in water-soluble vitamin capsules/tablets w/wo minerals	Method 1—HPLC 210 nm Method 3—HPLC 205 nm Method 2—Microbiological	None
3. Page 359	Calcium pantothenate	Calcium pantothenatetablets (NLT 95.0%, NMT 115.0%)	Microbiological	None
4. Pages 1633–1634	Panthenol	Panthenol (NLT 99.0%, NMT 102.0%)	Titration, perchloric acid	None
5. Pages 2427–2428, 2431, 2434–2435	Panthenol Dexpanthenol	Assay for dexpanthenol or panthenol	Method 1—HPLC 210 nm Method 2—Microbiological	None
***AOAC Official Methods of Analysis,* 18th ed., 2005**[70]				
1. 45.2.05	Pantothenic acid	AOAC Official Method, 945.74, Pantothenic Acid in Vitamin Preparations	Microbiological	*J. Assoc. Off. Anal. Chem.*, 42, 529, 1959;[71] *J. Biol. Chem.*, 192, 181, 1951[72]
2. 50.1.22	Calcium pantothenate	AOAC Official Method, 992.07, Pantothenic Acid in Milk-Based Infant Formula	Microbiological	*J. AOAC Int.*, 76, 398, 1993[73]
***Food Chemicals Codex,* 5th ed., 2004**[74]				
1. Page 133–134	DL-Panthenol	DL-Panthenol	Titration, perchloric acid	None

reference. Background information on the liberation of pantothenic acid from biologicals is provided in References 78–83.

13.3.2 Radioimmunoassay and enzyme-linked immunosorbent assay

A radioimmunoassay (RIA) for pantothenic acid was developed by Wyse et al.[84] for analysis of biological samples. Pantothenic acid was extracted by the "double-enzyme" digestion used for the *L. plantarum* assay. Results were shown to compare closely to the microbial assay.[85,86] The RIA was used to assay 75 processed and cooked foods.[86] Findings indicated relatively low amounts of pantothenic acid in some highly processed foods. Nonfortified cereals and fruit products were low in pantothenic acid compared to most other foods. All fruit products contained less than 0.2 mg per serving. Boiled or baked potatoes and canned tomato products contained higher levels of the vitamin. This study, to date, is one of the most comprehensive investigations completed on the pantothenic acid content of the U.S. food supply. In addition, RIA was applied to human milk.[87]

The RIA procedure developed by Wyse et al.[84] remains a viable alternative to the *L. plantarum* assay. This assay, like RIA methods for other water-soluble vitamins, has not been used extensively by other research groups.

Smith et al.[88] developed the first direct enzyme-linked immunosorbent assay (ELISA) for pantothenic acid. Antibodies specific for pantothenic acid were covalently linked to alkaline phosphatase with glutarylaldehyde. Immobilized pantothenate substrate was formed by attaching human serum albumin–pantothenate conjugate to the surface of polystyrene tubes by passive absorption. The binding of the enzyme-linked antibody to immobilized substrate is inhibited by free pantothenic acid in the standards or samples. Binding ratios were determined by the extent of hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase. In 1988, Morris et al.[89] reported an indirect double-antibody ELISA that was highly specific for pantothenic acid. The assay was specifically applied to food analysis.[90] Results compared closely to microbiological assay of *L. plantarum*. Song et al.[91] developed an indirect ELISA for plasma analysis with good results. The method requires no preparation of the plasma and was reported to be easier to use, less expensive, and more sensitive than other RIA or ELISA procedures for assay of free pantothenic acid. More recently, Gonthier et al.[92] evaluated various conjugates for their ability to raise antisera against pantothenic acid in rabbits. Using adipoyl dichloride and bromoacetyl bromide, only the acetyl conjugate induced production of a specific antibody. The developed ELISA had greater sensitivity compared to earlier ELISA methods. The use of pantothenic acid coupled to thyroglobin with adipoyl dichloride as the capture antigen was believed to increase the sensitivity of the assay. Finglas and Morgan[93] reviewed existing biospecific methods for water-soluble vitamins. This review provides excellent background material for the overall area.

RIA and ELISA tests are sensitive, less labor intensive, and provide acceptable alternatives to the microbiological assay of pantothenic acid in biological samples. The method of choice depends on availability of equipment, budget, and expertise available. RIA and ELISA tests require pantothenic acid to be in the free state, and enzyme digestion is necessary if total pantothenic acid is to be determined.

13.3.3 Advances in the analysis of pantothenic acid

Significant advances have occurred in the past decades in the application of capillary electrophoresis (CE), gas chromatography linked to mass spectrometry (GC-MS), LC-MS, and optical sensor immunoassay to the assay of pantothenic acid in foods, supplements, and biologicals. These methods are discussed in the following sections and selected methods are summarized in Table 13.5.

Table 13.5 Selected Capillary Electrophoresis, Gas Chromatography, Liquid Chromatography, and Optical Biosensor Immunoassay Methods for the Analysis of Pantothenic Acid

Matrix	Description	Accuracy/precision	References
Capillary electrophoresis			
Soft drinks	Chiral separation of native DL-pantothenic acid enantiomers by CE using 2-hydroxypropyl-β-dextrin as the chiral selector. Optimum running conditions were 60 mM 2-hydroxypropyl-β-cyclodextrin and 10% methanol, +20 kV at 15°C, 200 nm.	%Recovery—100 %RSD (peak area)—0.7	*J. Chromatogr. A*, 811, 269, 1998[98]
Soft drinks, supplements	Separation of six water-soluble vitamins was achieved with CE. 50 cm × 75 µm uncoated silica, borate buffer (25, 50, 75, 100 mM), pH 8.5 with different concentrations of SDS, +30 kV, 214 nm	DL—2.0 µg mL^{-1} QL—7.0 µg mL^{-1} %Recovery—48–61	*Nahrung/Food*, 47, 243, 2003[99]
Milk formulas, corn flakes	Column-coupling capillary isotachophoresis preseparation column—16 cm × 0.8 mm, analytical column–16 cm × 0.8 mm, fluorinated ethylene-propylene copolymer buffers Leading electrolyte—10 mmol L^{-1} HCl with 0.1% polyvinylpyrrolidone adjusted to pH 6.0 with histidene Terminating electrolyte—5 mmol L^{-1} 4-morpholine ethanesulfonic acid adjusted with TRIS to pH 6.2, driving current (preseparation) = 350 µA, analytical = 50 µA, detection—conductivity	%RSD—2.7–3.4 %Recovery—96–103 DL—0.10–0.13 mg L^{-1} QL—0.3–0.4 mg L^{-1}	*Eur. Food Res. Technol.*, 216, 440, 2003[100]
Model system	Chiral separation of DL-pantothenic acid enantimers was achieved by ligand exchange CE. Borate complexes containing (S)-3-amino-1,2-propanediol (SAP) and DL-pantothenic acid were resolved. Fused silica bubble cell capillary (64.5 cm × 75 µm), 200 mM SAP–200 mM borate, pH 9.2, containing 15% MeOH, +25 kV, 200 nm.	—	*Electrophoresis*, 27, 880, 2006[101]

Continued

Table 13.5 (Continued)

Matrix	Description	Accuracy/precision	References
Gas chromatography-mass spectrometry			
Plasma, brain, foods	Mass fragments of TMS-derivatives were quantified, chromatography – DB-17	DL — 1 ng mL^{-1}	*J. Chromatogr.*, 525, 225, 1990;[108] *Meth. Enzymol.*, 279, 213, 1997[110]
Plasma	After methyl esterification, D- and L-enantimers were derivatized by trifluoroacetate, cyclic sulfite or cyclic *n*-butylboronate. Mass fragments were quantified following resolution on Chirasil-D-val.	DL — 5 ng	*J. Chromatogr.*, 564, 1, 1991[109]
Foods	GC-MS of TMS derivatives of pantothenic acid and the internal standard calcium [^{15}N,$^{13}C_3$]-(R)-pantothenate provided a stable isotope dilution assay for free and bound pantothenic acid.	%Recovery — 97.5–99.4 DL — 44 µg kg^{-1} for starch	*J. Agric. Food. Chem.*, 48, 1175, 2000;[111] *J. Food Compos. Anal.*, 15, 399, 2002[112]
Liquid chromatography			
Infant formula	Reversed-phase chromatography Superesphere C_{18} (25 cm × 4.6 mm), 0.25 M sodium phosphate — acetonitrile (97 : 3), pH 2.5, 1 mL min^{-1}, 197 nm	%Recovery — 89–98 %CV — 1.2–3.2	*J. Dairy Sci.*, 79, 523, 1996[116]
Milk and infant formulas	Reversed-phase chromatography Luna C_8 (25 cm × 4.6 mm), 0.1 M phosphate, pH 2.5 — acetonitrile (970:30), 1–2 mL min^{-1}, multi-channel detection at 200, 205 and 240 nm	%Recovery — 99–99.8 $\%RSD_R$ — 4.9	*Food Chem.*, 69, 201, 2000[117]
Foods	Reversed-phase chromatography Postcolumn derivatization to form 1-alkylthio-2-alkylisoindole, fluorescence detection (Ex λ = 345, Em λ = 455). Lichrosphere 100 RP 18 (25 cm × 5 mm), Gradient elution 33 mM phosphate, pH 2.5 with linear increase of MeOH from 0% to 10% over 25 min	%Recovery — 96–101 $\%RSD_r < 8$	*J. Chromatogr. A*, 1035, 87, 2004[118]

LC-MS			
Foods	Stable isotope dilution Internal standard — [^{15}N,^{13}C] pantothenic acid Reversed-phase chromatography Aqua C_{18} (25 cm × 4.6 mm, 5 µm) Gradient elution A — 0.1% formic acid B — acetonitrile Flush for 9 min (93% A, 7% B); 13 min linear gradient to 17% B, 3 min linear gradient to 25% B, raise to 100% B, hold 5 min. LC-MS/MS mass transitions (m/z precursor ion/m/z product ion) labeled = 224/206, unlabeled = 220/202	%Recovery — 103 DL — 86 µg kg^{-1} QL — 240 µg kg^{-1} $\%CV_{Intra}$ — 4.5 $\%CV_{Inter}$ — 6.5	*Analyst*, 128, 832, 2003;[119] *Anal. Chim. Acta*, 495, 133, 2003[120]
Urine, foods	Internal standard — hopantenic acid Reversed-phase chromatography Urine Ace 3AQ C_{18}, 15 cm × 3.0 mm Gradient elution A — 0.025% trifluoroacetic acid, pH 2.6 B — MeCN:A (1:99) 100%A — 3 min; to 65% A:35% B in 20 min; 100% A — 11 min LC-MS-ESI, SIM mode	%Recovery ≥95.8 DL — 0.1 µg mL^{-1} QL — 0.25 µg mL^{-1}	*J. Sep. Sci.*, 28, 669, 2005;[121] *J. Chromatogr. A*, 1032, 1, 2004[122]
Optical biosensor immunoassay			
Foods	Qflex[R] kit — Biacore International Surface plasmon resonance	%Recovery >95% DL — 4.4 ng mL^{-1} $\%RSD_R$ — 6.3-26.4 HORRAT — 0.82–2.9	*J. AOAC Int.*, 88, 1008, 2005[123]

13.3.3.1 Capillary electrophoresis

Trenerry,[94] in an excellent review of the application of CE to assay of vitamins in food and beverages, reported only three methods[95–97] applied to analysis of pantothenic acid. Lack of sensitivity and presence of interferences with ultraviolet (UV) detection has limited the methods' use for pantothenic acid. Nevertheless, recent methods show promise.[98–101] Schreiner et al.,[99] using conventional CE techniques, presented a multivitamin assay, including pantothenic acid for the analysis of soft drinks and supplements. The detection limit for pantothenic acid was 2.0 μg/mL, which was about 10 times higher than many of the other vitamins included in the assay. Accuracy measured as %recovery was inadequate for pantothenic acid. The authors attributed the insufficiency of the overall method for pantothenic acid assay to the weak spectral properties of the vitamin.

Sádecká et al.[100] successfully assayed pantothenic acid in fortified and nonfortified foods using column-coupling capillary isotachophoresis. The limit of quantitation was 1.6 mg/kg, and the simplicity of the procedure led the authors to conclude its suitability for assay of low concentration foods. Chiral separation of DL-pantothenic acid enantiomers has been achieved by Kodama et al.[98] and Aizawa et al.[101] Kodama et al. used 2-hydroxypropyl-β-cyclodextrin as a chiral selector and analyzed pantothenic acid in a soft drink. While D-pantothenate was the only enantiomer present in the drink, spiking studies showed that both D- and L- forms could be quantified. Aizawa et al.[101] used ligand exchange CE to identify binary and ternary complexes that exist in a borate, (*S*)-3-amino-1,2-propanediol and DL-pantothenic acid ternary system at pH 9.2. Differences in electrophoretic mobility of the ternary complexes were shown to be due to differences in solvated structure.

13.3.3.2 Gas chromatography and gas chromatography-mass spectrometry

Early applications of gas chromatography (GC) to analysis of pantothenic acid were limited to high concentration pharmaceuticals or in some cases, relatively simple matrix biologicals (urine).[102–107] Volatile derivatives were formed through esterification of pantothenic acid or the assay of pantoyl lactone formed by acid hydrolysis of the sample with hydrochloric acid. More recent methods summarized in Table 13.5 rely on GC-MS.[108–112]

Rychlik[111] and Rychlik and Freisleben[112] developed a stable isotope dilution assay for free and bound pantothenic acid in foods and plasma that provides data comparable to data obtained by microbiological assay. The method uses a fourfold labeled isotopomer, calcium [^{15}N,$^{13}C_3$]-(*R*)-pantothenate, as the internal standard. GC-MS was used to quantify the trimethylsilyl derivatives of the vitamin and the labeled internal standard. Excellent recoveries and intrasample %RSD values show the strength of the assay. Mass chromatography of m/z 291 and 295 were used to compute ion abundance ratios (Figure 13.3).

13.3.3.3 Liquid chromatography and liquid chromatography-mass spectrometry

Liquid chromatography (LC) methods for pantothenic acid were hampered by the lack of UV absorption above 230 nm and difficulty in formation of reproducible derivative such as labeling with fluorescamine. Poor sensitivity of early methods, therefore, limited their application to high potency supplements.[113,114] Useful methods have been developed for relatively high concentration foods such as diet and milk powders and infant formulas.[115–117] Iwase[115] introduced a column-switching LC method for assay of pantothenic acid in commercial, elemental diets. Diet preparations were dissolved in water at 50°C. Sodium chloride was added and the solution was diluted with water. After hexane extraction, aliquots of the aqueous phase were injected. Chromatography included precolumn fractionation on Capcellpak C_{18} (150 cm × 4.6 mm) with switching to a Capcellpak C_{18} (25 cm × 4.6 mm) analytical column. The precolumn was eluted with acetonitrile:water, pH 2.1, (5:95). After washing with the mobile phase, adsorbed compounds were introduced onto the analytical

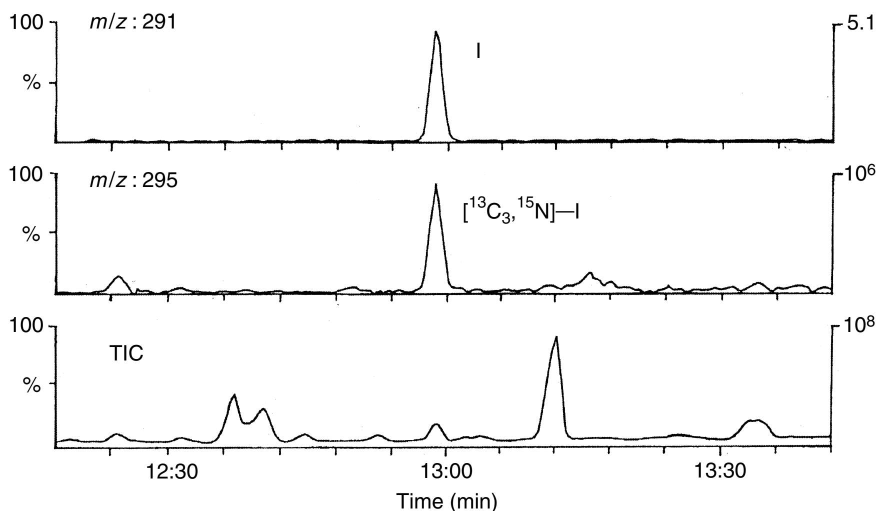

Figure 13.3 GC/mass chromatogram of unpolished rice containing 5.56 mg kg^{-1} of free pantothenic acid. The internal standard tris(trimethylsilyl)-[^{15}N,$^{13}C_3$]-pantothenic acid ([^{15}N,$^{13}C_3$]-I) is detected in the trace *m/z* 295, unlabeled tris(trimethylsilyl)pantothenic acid (I) in trace *m/z* 291. TIC: total ion current. (Reproduced from Rychlik, M. and Freisleben, A., *J. Food Compos. Anal.*, 15, 399, 2002. With permission.)

column with acetonitrile:water, pH 2.1, (9:91), containing 1.5 mM sodium 1-heptane sulfonate. Detection was at 210 nm. Recovery was 95% and within-day RSD was low at 1.8%. While the procedure gives excellent results, the complexity of the LC setup shows the problems inherently present in applying LC to the analysis of pantothenic acid. Romera and Gil[116] applied reversed-phase chromatography to analysis of pantothenic acid in infant formulas. Powdered formulas were homogenized in water at 40°C, cooled, and treated with acetic acid and sodium acetate in precipitate protein. After centrifugation, the supernatant was filtered and injected directly onto the LC column. Chromatography was completed on C_{18} with a mobile phase of 0.25 M sodium phosphate:acetonitrile, pH 2.5 (97:3) with detection at 197 nm. Recovery ranged from 89% to 98%. Coefficients of variation ranged from 1.3% to 3.2%. The procedure was stated to be an ideal replacement for the microbiological assay of pantothenic acid in infant formula. The simplicity of the procedure indicates that it could be widely applied to infant formula, medical foods, and other fortified foods. Also applicable to milk and infant formulas, Woollard et al.[117] used a simple deproteination of the matrix and LC analysis on a Luna C_{18} column to assay free pantothenic in the formulas. The assay provided "a reasonable estimate of total vitamin content in view of the minor contribution of coenzyme-bound pantothenate."

More recently, Pakin et al.[118] introduced a method for nonfortified foods using postcolumn derivatization of pantothenic acid to the fluorescent 1-alkylthio-2-alkylisoindole. This product forms when pantothenic acid is hydrolyzed to β-alanine under hot, alkaline conditions and, then, reacted with orthophthaldialdehyde in the presence of 3-mercaptopropionic acid. Recoveries with a variety of foods ranged from 96% to 101% and RSD_r was less than 8%.

Quite recently, Rychlik[119,120] modified the stable isotope dilution method originally developed for GC-MS assay of pantothenic acid[111,112] to an LC-MS/MS method. The LC-MS/MS procedure provided excellent performance data and should, when applied to food composition studies, provide excellent data. LC-MS procedures for quantification of

pantothenic acid in urine[121] and foods[122] were introduced by Heudi's research group at the Nestlé Research Center in Lausanne, Switzerland that incorporated hopantenic acid as the internal standard. Urine required only centrifugation and dilution (1:10) with 0.025% trifluoroacetic acid, pH 2.6, before injection. Food product homogenates were autoclaved in water, diluted with water, and filtered through a 0.22 µm filter before injection. Short analysis times, simplicity, and excellent performance parameters, as noted for other vitamins when assay with LC-MS, show the advantages of MS-based analysis of vitamins in foods and other biologicals. Liquid chromatography methods are summarized in Table 13.5.

13.3.3.4 Optical biosensor immunoassay

Haughey et al.[121] reported on the application of an optical biosensor inhibition immunoassay based on the Biacore International Qflex kit for pantothenic acid. The assay is an affinity-based immunosensor technique using surface plasmon resonance that detects the nonlabeled interaction of specific binding protein with immobilized ligand on a carboxymethyl dextran-modified gold sensor surface. The method follows the same principles used for the Qflex kits for folate and biotin. The optical detector measures changes in the resonance angle due to the molecular interaction at the interface. Such biosensor assays are discussed for folic acid (Chapter 10), vitamin B_{12}, a protein binding assay, (Chapter 11), and biotin (Chapter 12).

For pantothenic acid, excellent performance parameters are obtained. Further, data compared favorably with microbiological and LC-MS methods for several food matrices. The biosensor assay offers advantages of speed, specificity, and simplicity for food analysis.

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chapter fourteen

Multianalyte methods for analysis of the fat- and water-soluble vitamins

14.1 *Fat-soluble vitamins*

With acceptance of liquid chromatography (LC) in the 1970s as the primary technique for the analysis of fat-soluble vitamins in pharmaceuticals and biologicals, efforts to develop methods capable of simultaneously measuring two or more vitamins became common. Implementation of multianalyte procedures can result in assay efficiency with savings in time and materials. To be useful, a simultaneous assay must not lead to loss of assay sensitivity, accuracy, and precision when compared to methods that accurately quantify single vitamins.

Difficulty exists for multianalyte fat-soluble vitamin analysis because of the complexity of the analytes. Specific problems include

1. The vitamins are present in nature as structurally related vitamers.
   A. Carotenoids represent a truly challenging analytical area owing to the numbers and diversity present in fruits and vegetables. Often, *cis*-isomers must be resolved from the natural *trans*-isomers (Chapter 1).
   B. Vitamin E includes eight homologs that vary in biological activity (α-, β-, γ-, and δ-tocopherol and the corresponding tocotrienols) (Chapter 3).
   C. Vitamin D analysis is complicated owing to the potential presence of pre-D forms and structurally related sterols (Chapter 2).
2. The presence of synthetic forms or metabolites can complicate quantitation.
   A. Milk is fortified with retinyl palmitate and total vitamin A activity includes natural retinol and provitamin A carotenoids. *cis*-Isomers vary in biological activity from the *trans*-isomers (Chapter 1).
   B. All-*rac*-α-tocopheryl acetate is the primary fortification form of vitamin E. After saponification, all-*rac*-α-tocopherol cannot be resolved from *RRR*-α-tocopherol. The biological activity of all-*rac*-α-tocopherol is only 74% that of the natural vitamin. Therefore, accurate methods must quantitate the synthetic form in addition to the natural forms of vitamin E (Chapter 3).
   C. Fortified foods can contain both vitamin D_2 and vitamin D_3 (Chapter 2).
3. Concentrations of the various vitamins differ considerably in biologicals, pharmaceuticals, foods, feeds, and fortified products. Even in fortified foods, vitamin D is present at such low concentrations that extensive cleanup and/or concentration is often necessary before determinative assay.

4. Each fat-soluble vitamin requires specific detection parameters. No one detection mode is ideal for all of the vitamins. Simultaneous detection requires multiple detectors in series, programmable detectors, sensitive photodiode array detectors (PDA), or combinations of such detectors. Utilization of mass spectroscopy (MS) in combination with LC offers many advantages.
5. Stability differences among fat-soluble vitamins often complicate extractions. Vitamin K cannot withstand saponification.
6. Coeluting interferences often lead to the need for specialized sample cleanup approaches.
7. Initial setup costs can be expensive.
8. Fat-soluble vitamins are often encapsulated to enhance stability. Often, this factor can cause difficulty at the extraction stage. The literature does not adequately approach this problem at this point in time.

A single method that is universally applicable to the analyses of vitamins A, D, E, and K, their synthetic forms, and metabolites is still not routinely available. Increased capability of UV/visible, fluorescence, and PDA detectors have greatly influenced this area over the past decades. With the development of liquid chromatography-mass spectrometry (LC-MS) techniques and improvements in column resolution powers (C_{30} supports), useful methods are available to complete simultaneous assays of fat-soluble analytes. This chapter provides information on applications of multianalyte fat-soluble assay methods for several matrices of interest to the vitamin chemist.

Summaries of published multianalyte procedures are provided in Tables 14.1,[1–32] 14.2, and 14.3. Because of the diversity of detection systems used in the methods, the procedures are chronologically categorized by detection methods. Also, the tables are not inclusive of the many citations available and are meant to give an overview of the instrumentation, support, and detector capability available for separation of multi–fat-soluble vitamins from complex matrices.

14.1.1 Butter, margarine, and fats and oils

Margarine is a primary vehicle for the delivery of vitamin A activity and provides a significant source of vitamin E in diets where it is commonly used. It is fortified with retinyl palmitate, and/or in some cases, with retinyl acetate, and colored with β-carotene, which provides additional vitamin A activity. Because of the significance of the product, it was one of the earliest foods for which multianalyte methods were developed. Thompson and Maxwell[32] provided a method to assay retinol and β-carotene in margarine following saponification and hexane extraction. This procedure used different LC systems and separate injections, representing a concurrent assay. The first simultaneous method to assay total vitamin A in margarine was provided by Landen and Eitenmiller[2] in 1979. The method also introduced the use of high performance gel permeation chromatography (HP-GPC) to isolate the fat-soluble vitamins from the lipid fraction before determinative chromatography. This approach allowed simultaneous chromatography of β-carotene and quantitated the specific vitamin A forms in the food. Thompson et al.[33] also presented a simultaneous analysis of retinyl palmitate and β-carotene in margarine that avoided saponification. The method was based on direct extraction of the analytes with hexane. Five grams of sample were dissolved in hexane, insoluble material was allowed to settle, and 5 mL of the clear solution was shaken with 5 mL of 60% ethanol. After centrifugation, retinyl palmitate and β-carotene were quantified by direct injection of the extraction onto a Si60 column using ethyl ether:hexane (2:98) for elution. Retinyl palmitate and β-carotene were detected at 325 and 453 nm, respectively. It is highly reliable for routine vitamin A analysis of margarine and fluid milks. A study by Zonta and Stancher[7] published in 1982 was one of the first simultaneous analysis

Table 14.1 Selected LC and LC-MS Methods for Multianalyte Assay of Fat-Soluble Vitamins in Foods and Feeds

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	References
UV/VIS and/or fluorescene				
Infant formulas, dairy products/ vitamins A, D_2 or D_3, E, and K_1	Vigorously stir sample (containing 3.5 ~ 4.0 g fat) w/200 mL buffer, pH 7.7 and 2.5 g lipase, 37°C, 1h. Saponify the extract w/10 N NaOH. Extract w/3× *n*-pentane. Wash combined extracts w/water. Filter the extracts through anhydrous sodium sulfate and evaporate to dryness	Two Zorbax ODS, 25 cm × 4.6 mm connected in series Mobile phase—gradient MeOH–EtOAC–MeCN Flow rate—gradient	325 and 365 nm for vitamin A forms; 265 nm for the rest	*Anal. Chem.*, 52, 610, 1980[1]
Infant formulas, margarine, oils, milk, cereal/ vitamins A, E, D_2, D_3, K_1 and β-carotene	Homogenization in mixture of IPA and CH_2Cl_2 w/$MgSO_4$ Clean-up: fractionate vitamins from lipids by HP-GPC Four µStyragel columns in series, 100 Å	Zorbax ODS. 6 µm, 25 cm × 4.6 mm Mobile phase—isocratic CH_2Cl_2:MeCN:MeOH (300:700:2) Flow rate—1 mL min^{-1}	325 nm for A 436 nm for β-carotene 280 nm vitamin D, E and K	*J. Assoc. Off. Anal. Chem.*, 62, 283, 1979;[2] 63, 131, 1980;[3] 65, 810, 1982;[4] 68, 509, 1985;[5] *J. Food Compos. Anal.*, 2, 140, 1989[6]
Foods/vitamins A, D_2, D_3, E, carotene	Saponify the sample and extract w/Hex. Evaporate the extract to dryness. Dissolve the residue w/MeOH	ODS-HC Sil-1 (25 cm × 2.6 mm) and Supelcosil LC18 (15 cm × 4.6 mm) in series Mobile phase—gradient MeOH—MeCN—water Flow rate—1.5 mL min^{-1}	Multiwavelength	*J. Chromatogr.*, 246, 105, 1982[7]
Pediatric parenteral/vitamins A, D_2, E and K_1	Extract w/Hex. Evaporate and dissolve in EtOH	Ultrasphere Si, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic 100 % MeOH Flow rate—0.2 mL min^{-1}	325 nm for A 265 nm for D_2 284 nm for E 250 nm for K_1	*J. Liquid Chromatogr.*, 17, 4513, 1994[8]

Continued

Table 14.1 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	References
Animal feed/ vitamins A, D, E, and pre-vitamin D_2	Vortex 1 g ground sample (particle size ≤80 mesh) w/4 mL A—$CHCl_3$ (30:70), 1 min. Rest 5 min, then vortex 0.5 min. Centrifuge, evaporate 1 mL supernatant to dryness. Redissolve in 0.3 mL *n*-butanol	Novapak C_{18}, 15 cm × 3.9 mm Mobile phase—isocratic 100% MeOH Flow rate—1.5 mL min^{-1}	290 nm	*J. Chromatogr. A*, 825, 127, 1998[9]
Dairy products/ vitamins A, D, E, and K	Mix sample w/EtOH containing BHT, sonicate, 2 min. Shake mixture w/Hex 2x in the Sep. funnel Wash combined Hex layer w/MeOH—water (9:1). Evaporate the extract to dryness, redissolve in MeOH	Hypersil C_{18} BDS, 3 µm, 15 cm × 0.3 mm Mobile phase—gradient MeOH–water–THF Flow rate—6 µL min^{-1})	325 nm for A 264 nm for D 280 nm for K and E	*Analyst*, 125, 427, 2000;[10] *J. Chromatogr. A*, 891, 109, 2000[11]
Animal products/ vitamins D, A, E and β-carotene	Saponify samples w/appropriate amount of KOH and antioxidant in boiling water bath, 20 min. Extract w/Hex:EtOAC (8:2), 2×. Evaporate combined extracts to dryness, redissolve in Hex Clean-up: semipreparative for vitamin D	NP (for A, E & β-carotene): µPorasil, 10 µm, 30 cm × 3.9 mm Mobile phase—gradient Hex–DIPE RP (for D): Nova-Pak C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic 93% MeOH in water	265 nm for D 325 nm for A 450 nm for β-carotene Ex λ = 285 Em λ = 310 for E	*Food Chem.*, 71, 535, 2000[12]
Fortified foods/ vitamin E, β-carotene, retinyl palmitate	Sonicate samples (3 g) w/hot water (2 mL, 80°C), add IPA and anhydrous $MgSO_4$. Extract w/Hex— EtOAC (9:1), 2x. Dilute combined extracts to 100 mL w/Hex. Evaporate 2 mL extract to dryness, redissolve in 2 mL mobile phase	LiChrosorb Si60, 5 µm, 25 cm × 4.6 mm Mobile phase—gradient Hex–IPA Flow rate—gradient	450 nm for β-carotene and fluorescence for E and A	*J. Agric Food Chem.*, 48, 4003, 2000[13]

Various foods/ vitamins A and E	Saponify homogenized sample w/KOH and antioxidant. Cool, neutralize the KOH w/glacial HAC. Dilute the extract w/THF—95% EtOH (1:1) to 100 mL. Let it set at least 1 h, before analysis.	RP C_{18}, 10 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH–water Flow rate—1 mL min^{-1}	313 or 328 nm for A and Ex λ = 290 Em λ = 330 for E	*J. AOAC Int.*, 85, 424, 2002[14]
ATBC drinks/ tocopherols and carotenes	Extract samples w/A—Hex (1:1), add sodium chloride solution (10%) to remove the emulsion. Wash Hex layer w/sodium chloride solution. Evaporate Hex extract to dryness. Redissolve in IPA.	YMC C_{30}, 5 µm, 25 cm × 4.6 mm Mobile phase—gradient MeOH–MTBE–water Flow rate—1 mL min^{-1}	Multiwavelength	*Food Chem.*, 76, 357, 2002[15]
Olive and soybean oils/ vitamin E and β-carotene	Dissolve sample in IPA–Hex (1.5:98.5)	Lichrosorb Si-60, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic IPA:Hex (1.5:98.5) Flow rate—1.0 mL min^{-1}	UV/VIS for β-carotene and fluorescence for vitamin E	*J. Food Sci.*, 68, 1644, 2003[16]
PDA and/or Fluorescence				
Vegetables, fruits and multivitamin tablets/retinoids, carotenoids, and tocopherols	Homogenize sample in IPA—CH_2Cl_2 (2:1), vortex 1 min and kept under argon overnight,—20°C. Vortex next day, 1 min, then return to the freezer. On third day, vortex, 1 min, centrifuge, filter, evaporate to dryness. Redissolve in IPA–CH_2Cl_2 (2:1)	Microsorb-MV C_{18}, 3 µm, 10 cm × 4.5 mm Mobile phase—gradient MeOH–water–CH_2Cl_2 containing 10 mM NH_4OAC Flow rate—0.8 mL min^{-1}		*J. Chromatogr. B*, 707, 69, 1998[17]
Virgin olive oil/ vitamin E, carotenoids	Dissolve sample in Hex:IPA (99:1) and filter	Lichrospher Si60, 5 µm, 25 cm × 4 mm Mobile phase—gradient Hex–IPA Flow rate—1.2 mL min^{-1}	294 nm for E Multiwavelength for carotenoids	*J. Agric. Food Chem.*, 46, 5132, 1998[18]

Continued

Table 14.1 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	References
Milk-based infant formula/ α-tocopheryl acetate, retinyl palmitate	Extract sample using matrix solid phase dispersion. Elute w/0.5% IPA in Hex and CH_2Cl_2. Evaporate the extract to near dryness, dilute to 1 mL w/Hex	Lichrosorb Si60, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic 0.125% IPA in Hex for retinyl palmitate 0.5% IPA in Hex for vitamin E Flow rate—1 mL min^{-1}	Ex λ = 285, Em λ = 310 for E and Ex λ = 325, Em λ = 470 for A	*J. AOAC Int.*, 81, 582, 1998[19]
Weaning foods/ vitamin E, retinyl palmitate	Homogenize 1.0 g sample w/hot water (80°C, 4 mL), add IPA and anhydrous $MgSO_4$ (5 g). Extract w/30 mL Hex: EtOAC (85:15), filter through a vacuum bell jar. Re-extract filter cake one more time. Dilute combined extracts to 100 mL w/Hex–EtOAC, filter	Lichrosorb Si60, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic 0.9% IPA in Hex Flow rate—1 mL min^{-1}	Ex λ = 285, Em λ = 310 for E and Ex λ = 325, Em λ = 470 for A	*J. Food Sci.*, 64, 968, 1999[20]
Infant formulas/ Retinol and α-tocopherol		Spherisorb ODS-2, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:water (95:5) Flow rate—1.0 mL min^{-1}	292 nm for α-tocopherol 325 nm for retinol	*Food Chem.* 66, 221, 1999[21]
Reduced fat mayonnaise/ β-carotene and vitamin E	Sonicate sample w/hot deionized water (80°C), add IPA and anhydrous $MgSO_4$. Extract w/Hex: EtOAC (90:10), 2x. Evaporate aliquot of combined extracts to dryness. Redissolve in mobile phase.	Lichrosorb Si60, 5 µm, 25 cm × 4.6 mm Mobile phase—gradient Hex–IPA Flow rate—gradient	220 ~ 500 nm	*J. Food Sci.*, 66, 78, 2001[22]

Shrimp waste/ retinol, α-tocopherol	Saponify the sample w/KOH (0.5 M in MeOH), 80°C, 15 min, and extract w/Hex. Evaporate the extract to dryness and redissolve in mobile phase	SS Exil ODS, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:MeCN:water (68:28:4) Flow rate—1.4 mL min^{-1}	325 nm for retinol 208 nm for α-tocopherol	*J. Chromatogr. A*, 1105, 135, 2006[23]
Animal meats/ α-tocopherol, β-carotene	Saponify the homogenized sample w/KOH (11% in 55% EtOH), 80°C, 15 min, and extract w/Hex. Filter	Zorbax RX-Sil, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic IPA:Hex (1:99) Flow rate—1 mL min^{-1}	PDA for β-carotene and fluorescence for vitamin E	*Food Chem.*, 94, 469, 2006[24]
Infant milk-based formula/vitamins A and E	Homogenized 2 g sample w/8 mL warm water (40°C). Add 3 mL EtOH and 1 mL Hex to 1 mL reconstituted sample, shake, 5 min. Add 3 mL saturated NaCl , centrifuge. Filter Hex phase.	Pinnacle II silica, 3 µm, 50 mm × 2.1 mm Mobile phase—isocratic EtOAC:Hex (0.5:99.5) Flow rate—0.4 mL min^{-1}	284 nm for α-Tocopherol acetate, 296 nm for Tocopherols and 326 nm for vitamin A	*J. Chromatogr. A*, 1122, 138, 2006[25]
EC				
Milk, milk powder/ α-T, all-*trans*-retinol, K_1	a. α-T, retinol—saponification, overnight, ambient. Extract w/Hex, evaporate, redissolve in MeOH. b. K_1—lipase hydrolysis, add alcoholic sodium hydroxide and immediately extract w/Hex. Clean-up: Sep-Pak silica for K_1	a. OD-224 RP18, 5 µm, 22 cm × 4.6 mm b. Brownlee OD-224 RP18, 5 µm Mobile phase—isocratic, a. 99% MeOH in water containing 2.5 mM HAC; b. 99% MeOH in water containing 2.5 mM HAC–NaOAC Flow rate—1.25 mL min^{-1}	EC Dual-amperometric glassy carbon –1100 mV +700 mV versus Ag/AgCl	*J. Chromatogr.*, 623, 69, 1992;[26] *Analyst*, 120, 2489, 1995[27]

Continued

Table 14.1 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	References
Milk/vitamins A, D_3, E	Saponify through on-line system which includes two confluent channels through which the sample solution and alcoholic sodium hydroxide plus ascorbic acid flowed for a given period of time. Neutralize the solution in the third channel before arrival at the C_{18} cartridge	OD-224 RP18, 5 µm, 22 cm × 4.6 mm Mobile phase—isocratic 99% MeOH in water containing 2.5 mM HAC–NaOAC Flow rate—1 mL min^{-1}	EC +1300 mV UV 280 nm	*J. Chromatogr. A*, 694, 399, 1995[28]
Vegetable oils/ vitamin E, carotenoids	Dissolve sample I n MTBE:MeOH (1:1), filter	YMC C_{30}, 5 µm, 25 cm × 4.6 mm Mobile phase—gradient MeOH–MTBE–NH_4OAC–water	EC 260 ~ 500 mV	*JAOCS*, 79, 633, 2002[29]
MS				
Fortified infant formula, cereal/ vitamins A, D_3, E	Homogenize sample w/warm water (40°C), saponify the portion of the mixture w/KOH, 85°C, 30 min. Extract the solution using SPE cartridge (Chromabond XTR®)	Nucleosil 100-5, 25 cm × 4.6 mm Mobile phase—isocratic Hex:Dioxan:IPA (96.7:3:0.3) Flow rate—1.45 mL min^{-1}	APCI Positive ion mode w/SIM	*J. Chromatogr. A*, 1022, 115, 2004[30]
Crude rice bran oil/carotenoids, tocopherols	Extract sample w/Hex, evaporate solvent. Disperse the refined oil in MeCN:MeOH:IPA (50:45:5). Centrifuge, filter the upper layer	Bischoff ProntoSIL 200-3-C_{30}, 3 µm, 25 cm × 2 mm Mobile phase—isocratic MeOH:TBME (75:25) Flow rate—0.2 mL min^{-1}	APCI Positive ion mode w/SIM	*J. Sep. Sci.*, 28, 1712, 2005[31]

Table 14.2 Selected LC and LC-MS Methods for Multi-Analyte Assay for Fat-Soluble Vitamins in Pharmaceuticals

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	References
UV/Vis and/or fluorescence				
Cosmetic creams/ retinyl palmitate, all-*rac*-α-tocopheryl acetate	Supercritical fluid extraction w/CO_2— a. Dynamic, 250 atm, 40°C, 30 min b. Restrictor 100°C, flow rate—190–200 mL min^{-1} Collect into THF:MeOH (4:1)	μBondapak C_{18}, 10 μm, 30 cm × 3.9 mm Mobile phase—isocratic MeOH:MeCN (75:25) Flow rate—1.5 mL min^{-1}	280 nm for α-tocopheryl acetate 325 nm for retinyl palmitate	*J. Pharm. Biomed.* Anal., 13, 273, 1995[38]
Multivitamin tablets/carotenoids, vitamins A, E	Add β-apo-8′-carotenal (IS) and 25 mL DMSO to well ground sample, sonicate 40°C, 20 min. Extract the mixture w/ Hex, 4x. Dilute the combined Hex layer to 200 mL w/Hex. Filter	Symmetry C_{18}, 5 μm, 15 cm × 4.6 mm Mobile phase—gradient MeCN—0.05% TEA in CH_2Cl_2— 0.25% NH_4OAC in MeOH Flow rate—1.5 mL min^{-1}	325 nm for retinol, 285 nm for Tocopherols and 450 nm for β-carotene	*J. AOAC Int.*, 82, 68, 1999[39]
Emulsified nutritional supplements/ vitamin A, β-carotene	Dissolve sample in 5% aqueous Na_2SO_4 solution containing 1 mM EDTA. Clean-up: Bond Elut C_{18}	Intersil ODS, 5 μm, 15 cm × 4.6 mm Mobile phase—isocratic MeOH:MeCN (30:70) Flow rate—1.6 mL min^{-1}	VIS and fluorescence	*Anal. Chim. Acta*, 463, 21, 2002[40]
Multivitamin preparations/water-, fat-soluble vitamins	Dissolve fat-soluble vitamins in 0.1% BHT in MeOH, held in dark, 2 h. Stir 20 min. Centrifuge.	Nucleosil 100-5, C_{18}, 75 mm × 2 mm Mobile phase—isocratic MeCN:water (95:5) Flow rate—100 μL min^{-1}	Multi-wavelength	*J. Anal. Chem.*, 57, 40, 2002[41]
Pharmaceuticals/ Vitamins D_3, K_1	Dissolve 0.05 g granulate sample in 1 mL water and extract w/1 mL Hex, 4×. Evaporate combined Hex extracts to dryness, redissolve in Hex	LiChroCart Si60, 5 μm, 25 cm × 4 mm Mobile phase—isocratic Hex–*n*-amyl alcohol Flow rate—1.0 mL min^{-1}	254 nm	*J. Biochem. Bioph. Methods*, 69, 101, 2006[42]

Continued

Table 14.2 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	References
PDA				
Pharmaceutical preparations/ 12 water- and fat-soluble vitamins	Extract sample w/EtOH, 2x for fat-soluble vitamins. Then, extract the residue w/KH_2PO_4 buffer (0.1 M, pH 7) for water-soluble vitamins. Combine the extracts and dilute w/EtOH:water (50:50). Filter and inject	µBondapak C_{18}, 10 µm, 30 cm × 3.9 mm Mobile phase—gradient MeOH—KH_2PO_4 buffer (0.1 M, pH 7)—water Flow rate—1.5 mL min^{-1}	Multi-wavelength	*Chromatographia*, 54, 270, 2001[43]
Capsules/fat-soluble vitamins	Dissolve the contents of one capsule in 10 mL Hex in a 100 mL volumetric flask, dilute to 100 mL w/MeOH. Place the solution in ultrasonic bath for 10 min at 30 °C	Lichrospher C_8, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:MeOH (95:5) Flow rate—2 mL min^{-1}	285 nm	*J. Liq. Chrom. Rel. Technol.*, 24, 201, 2001[44]
EC				
Pharmaceuticals/tocopherol, retinyl acetate	Dissolve sample in MeOH and deionized water, centrifuge. Filter supernatant and inject.	µBondapak C_{18}, 10 µm, 30 cm × 3.9 mm Mobile phase—isocratic MeOH:water (92:8) containing 0.1 M acetate buffer (pH 4.72) Flow rate—1 mL min^{-1}	EC+1000 mV vs Ag/AgCl	*J. Pharm. Biomed. Anal.*, 25, 785, 2001[45]
MS				
Botanical materials/α-tocopherol, carotenoids	Extract oil, fine dry material or aqueous sample w/Hex. Use anhydrous sodium sulfate to remove moisture. Extract wet hard botanical tissue w/MeOH:THF (1:1), evaporate the extract to dryness, redissolve in Hex.	Zorbax RX-SIL, 5 µm, 15 cm × 2.1 mm Mobile phase—gradient Hex–IPA–EtOAC Flow rate—0.5 mL min^{-1}	MS/MS-APCI Positive ion mode w/SRM	*J. Chormatogr. A*, 1094, 83, 2005[46]
Multivitamin dietary supplements/ vitamins A, E	Capsules—extract sample w/TBME: MeOH (1:1), 5x. Tablets—extract sample w/PE:EtOAC: MeOH (1:1:1), 5x. Add 2 mL EtOH to the combined extracts and evaporate in vacuum, water bath, 50°C. Dissolve residue w/TBME:MeOH (1:1).	YMC C_{30}, 5 µm, 25 cm × 4.6 mm Mobile phase—gradient MeOH:water:TBME Flow rate—1 mL min^{-1}	MS-APCI Positive ion mode w/SIM	*Eur. Food Res. Technol.*, 222, 643, 2006[47]

Table 14.3 Selected LC and LC-MS Methods for Multi-Analyte Assay of Fat-Soluble Vitamins in Serum and Tissues

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	References
UV/VIS and/or Fluorescence				
Plasma, serum/ all-*trans*-retinol, α-T, β- + γ-T, K_1	Add retinyl acetate (IS) and all-rac-α-tocopherol acetate (IS). Deproteinize w/EtOH. Extract w/Hex, wash Hex layer w/MeOH:water (9:1). Evaporate and dissolve residue in IPA	Resolve C_{18}, 5 µm, 15 cm × 3.9 mm Mobile phase—isocratic EtOH:water (92:8)	Fluorescence after reduction for K_1—Ex λ = 320 Em λ = 430 280 nm for the rest.	*Am. J. Clin. Nutr.*, 32, 2143, 1979;[49] *Clin. Chem.*, 35, 2285, 1989[50]
Serum/retinol, α-T, β-carotene	Mix sample w/EtOH and IS. Add Hex, vortex. Centrifuge, evaporate Hex layer to dryness. Dissolve the residue in mobile phase	Ultrasphere ODS, 5 µm, 15 cm × 4.6 mm Mobile phase—isocratic $MeCN:MeOH:CH_2Cl_2$ (70:10:20) Flow rate—1.2 mL min^{-1}	Multi-wavelength	*J. Chromatogr.*, 572, 103, 1991[51]
Human plasma, tissue/retinol, tocopherol, five carotenoids	Digest tissue samples w/enzyme, extract w/Hex, 2×. Add EtOH to plasma, mix and extract w/Hex, 3×. Centrifuge and evaporate the extract to dryness. Dissolve the residue w/EtOH and mobile phase	Ultracarb ODS, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic MeCN:THF:MeOH:1% NH_4OAC (65:25:6:4) Flow rate—1.7 mL min^{-1}	Multi-wavelength	*Am. J. Clin. Nutr.*, 56, 417, 1992[52]
Human serum/ nine carotenoids, retinol, retinyl palmiltate, α-T	Add α-tocopherol acetate (IS) and reinyl acetate (IS) in EtOH (0.8 mL) and EtOH (0.8 mL) to sample (0.8 mL), mix. Extract w/Hex (0.8 mL), mix. Extract w/Hex (2 mL), 2×. Evaporate supernatant to dryness, reconstitute w/THF	Spheri-5-RP-18, 5 µm, 22 cm × 4.6 mm Mobile phase—isocratic $MeCN:CH_2Cl_2:MeOH$ (70:20:10) Flow rate—1.8 mL min^{-1}	Multi-wavelength	*Food Chem.*, 45, 205, 1992[53]
Human serum/ carotenoids, retinyl, retinyl esters, tocopherols	Add IPA (2× volume of sample) to sample and extract w/CH_2Cl_2 (same volume of sample). Vortex, and centrifuge. Dilute the supernatant w/IPA:CH_2Cl_2 (2:1) if necessary	Waters C-18, 5 µm, 30 cm × 3.9 mm Mobile phase—isocratic $MeCN:CH_2Cl_2:MeOH$:water containing 0.1% NH_4OAC (90:10:5:2) Flow rate—1 mL min^{-1}	Multi-wavelength or PDA	*J. Chromatogr.* 617, 257, 1993;[54] *Food Chem.*, 46, 419, 1993[55]

Continued

Table 14.3 (Contineud)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	References
Human serum/ carotenoids, retinoids, tocopherols	Add EtOH, mix. Extract w/Hex, 2×. Evaporate combined supernatant to dryness, reconstitute w/EtOH	Bakerbond C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—gradient MeCN—MeOH—EtOAC	Multi-wavelength	*J. Chromatogr.*, 619, 37, 1993[56]
Serum/retinol, α-T, six carotenoids, four retinyl esters	Mix 200 µL EtOH containing IS w/200 µL serum, extract w/1 mL Hex. Dry Hex layer under reduced pressure to a waxy consistency. Dissolve the residue w/100 µL EtOH and 100 µL MeCN. Filter	Ultramex ODS, 5 µm, 15 cm × 4.6 mm Mobile phase—isocratic EtOH : MeCN (1:1) containing 0.01% diethylamine Flow rate—0.9 mL min^{-1}	Multi-wavelength	*Clin. Chem.*, 40, 411, 1994[57]
Human serum/retinol, α-T, 25(OH)D_2, 25(OH)D_3	Mix 500 µL sample w/50 µL EtOH containing IS, incubate for 10 min, room temperature. Add 350 µL MeOH:IPA (80:20) and extract w/Hex (1.5 mL). Centrifuge, evaporate Hex layer to dryness, redissolve in MeOH	Supelcosil LC-18, 5 µm, 25 cm × 4.6 mm Mobile phase—gradient MeOH—water Flow rate—2.5 mL min^{-1}	Multi-wavelength	*J. Ped. Gastr. Nutr.*, 18, 339, 1994[58]
Plasma/vitamins K_1, A, E, β-carotene	Add dihydrovitamin K_1, EtOH. Extract w/Hex, centrifuge, collect Hex layer. Clean-up: partition Hex w/MeOH:water (9:1)	Hypersil ODS, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic $MeOH:CH_2Cl_2$ (9:1) containing $ZnCl_2$, NaOAC and HAC Flow rate—1.0 mL min^{-1}	VIS/UV and fluorescence	*Int. J. Vit. Nutr. Res.*, 65, 31, 1995[59]
Human, Bovine serum/vitamins A, D, E, K	Add 5 mL EtOH to 5 mL serum, mix and centrifuge. Extract 2 mL of the serum w/3 mL Hex, 1 mL EtOAC. Wash the organic layer w/MeOH-water (9:1). Evaporate supernatant to dryness, redissolve in MeOH	Hypersil C_{18}, 25 cm × 4.6 mm Mobile phase—isocratic $MeCN:CH_2Cl_2:MeOH$ (60:20:20) Flow rate—1.0 mL min^{-1}	Multi-wavelength	*Chromatographia*, 55, 289, 2002[60]

Rat plasma/ retinol, α-, γ-T	Mix 200 μL sample w/200 μL EtOH, 200 μL MeOH, extract w/1 mL Hex, 2×, and centrifuge. Evaporate combined supernatant to dryness. Dissolve residue w/MeOH	Supelco Discovery C_{18}, 5 μm, 15 cm × 4.6 mm Mobile phase—isocratic MeOH:water (95:5) Flow rate—2 mL min^{-1}	UV—340 nm, 295 nm Fluorescence Ex λ = 295 nm Em λ = 350 nm	*J. Chromatogr. B*, 800, 225, 2004[61]
Human plasma/ retinol, α-T	Add 0.5 mL MeOH to 0.25 mL sample, mix and extract w/Hex (0.75 mL), centrifuge. Remove Hex layer and extract sample w/Hex again. Evaporate combined Hex extracts to dryness. Dissolve residue w/0.25 mL mobile phase	LiChrospher 100 RP-18, 5 μm, 12.5 cm × 4 mm Mobile phase—isocratic Hex:MeOH (28:72) Flow rate—1 mL min^{-1}	Multi-wavelength	*J. Pharm. Biomed. Anal.*, 42, 232, 2006[62]
PDA and/or fluorescence				
Serum, tissues, plants/α-T, γ-T, 20 carotenoids, 5 retinoids	Add EtOH, extract w/Hex. Evaporate to dryness. Redissolve residue in CH_2Cl_2.	Vydac 201 TP54, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic MeOH:MeCN (9:1) Flow rate—1.0 mL min^{-1}	PDA 200–800 nm	*J. Liq. Chromatogr.*, 18, 2813, 1995[63]
Adipose tissue/ retinol, β-carotene, α-T	Homogenize EtOH and saturated NaCl (200 μL each) containing AA w/32.5 mg sample. Extract w/solvent (2% IPA in Hex, 0.01% TBHQ, 500 μL), 3×, vortex and centrifuge. Evaporate combined supernatant to dryness; redissolve in 0.7% IPA in Hex	LiChrosorb Si60, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient Hex–IPA Flow rate—1 mL min^{-1}	PDA and fluorescence	*J. Chromatogr. B*, 763, 1, 2001[64]
Plasma/ retinol, α-T	Mix 200 μL sample w/200 μL EtOH, 200 μL retinol acetate (IS). Add 200 μL water and 500 μL Hex to the mixture, mix and centrifuge. Remove 300 μL supernatant, extract the rest of solution w/300 μL Hex. Evaporate combined supernatant to dryness, dissolve the residue w/MeOH:EtOH:Hex (88:10:2).	Supelcosil, 3 μm, 75 mm × 4.6 mm Mobile phase—isocratic 100% MeOH Flow rate—1.3 mL min^{-1}	PDA 292 nm 325 nm	*J. Pharm. Biomed. Anal.*, 28, 991, 2002[65]

Continued

Table 14.3 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	References
Human serum/ five carotenoids, retinol, α-T	Add 300 μL IS in EtOH and 200 μL EtOH to 200 μL sample, vortex. Add 1 mL water and extract w/2 mL Hex containing 0.01% BHT. Centrifuge and evaporate organic layer to dryness. Redissolve residue w/THF	Nucleosil C_{18}, 5 μm, 25 cm × 3 mm Mobile phase—isocratic MeOH:MeCN:THF (75:20:5) Flow rate—0.6 mL min^{-1}	PDA 290 nm 325 nm 450 nm	*J. Chromatogr. Sci.*, 40, 69, 2002[66]
Human plasma/ vitamins C, E, β-carotene	For vitamin E, β-carotene—Mix 100 μL EtOH w/100 μL sample. Extract w/ 600 μL Hex, and centrifuge. Evaporate the organic layer to dryness, redissolve in MeOH	LiChrospher 100-RP18, 5 μm, 12.5 cm × 4 mm Mobile phase—isocratic MeOH:MeCN:THF (75:20:5) Flow rate—1.2 mL min^{-1}	PDA 200–400 nm	*J. Pharm. Pharmaceut.* Sci., 7, 200, 2004[67]
Serum/retinol, α-T, $25(OH)D_3$, 24 R, $25(OH)D_3$	Add IS (20 μL) to 1 mL sample and mix the sample w/800 μL EtOH. Extract the sample w/4 mL Hex:CH_2Cl_2 (90:10), 2×. Centrifuge and evaporate combined extracts to dryness, redissolve in MeOH	Ultrabase C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient MeOH—IPA Flow rate—gradient	PDA 265 nm 291 nm 325 nm	*J. Steroid Biochem.* Mol. Biol., 89–90, 473, 2004[68]
Human serum/ retinol, α-T	Deproteinized 500 μL serum w/500 μL 5% MeOH in EtOH. Extract the sample w/2.5 mL Hex, centrifuge. Evaporate the clean extract to dryness. Redissolve in MeOH	Monolithic column RP-18e, 5 μm, 10 cm × 4.6 mm Mobile phase—isocratic, 100% MeOH Flow rate—2.5 mL min^{-1}	PDA 295 nm 325 nm	*Anal. Chim. Acta*, 573–574, 267, 2006[69]

Human Serum/ retinol, retinyl esters, α-T	Shake 500 μL sample w/2.5 mL Hex: T (8:2), 5 min. Add 500 μL cool EtOH w/5% MeOH (4°C, 5 min). Centrifuge, 0°C, 10 min. Collect 2 mL clear extract, extract the rest of serum sample w/2 mL Hex:T (8:2), again. Evaporate combined extracts and dissolve the residue w/100 μL Hex and 300 μL MeOH	Chromolith Performance RP-18e, 10 cm × 4.6 mm Mobile phase—gradient MeOH–water–IPA Flow rate—3 mL min^{-1}	PDA 325 nm 295 nm 330 nm	*J. Sep. Sci.*, 29, 2485, 2006[70]
EC				
Rat plasma/ retinol, α-, γ-T	Mix 200 μL sample w/200 μL EtOH, 200 μL MeOH, extract w/1 mL Hex, 2×, and centrifuge. Evaporate combined supernatant to dryness. Dissolve residue w/MeOH	Supelco Discovery C_{18}, 5 μm, 15 cm × 4.6 mm. Mobile phase—isocratic MeOH:water (95:5) containing 50 mM NaOAC, 150 mM $LiClO_4$ Flow rate—2 mL min^{-1}	EC Glass carbon 700 mV versus Ag/AgCl	*J. Chromatogr*. B, 800, 225, 2004[71]
MS				
Serum/carote-noids, retinol, vitamin K	Grind sample under liquid nitrogen. Saponify sample w/KOH and extract w/Hex:T (10:8), 2×. Evaporate to dry-ness, redissolve in MTEB:MeOH (1:1)	YMC C_{30}, 3 μm, 25 cm × 4.6 mm Mobile phase—gradient 1 mM NH_4OAC–MeOH–MTEB Flow rate—1 mL min^{-1}	APCI Positive ion mode w/SIM	*J. Agric. Food Chem.*, 51, 4877, 2003[72]
Serum/retinol, α-T, β-carotene	Add 240 μL EtOH:EtOAC (1:1) to 60 μL serum sample, vortex and centrifuge. Inject 20 μL of the denatured sample	Supelcosil LC-8-DB, 3 μm, 15 cm × 4.6 mm Mobile phase—isocratic MeOH:CH_2Cl_2 (95:5) Flow rate—0.8 mL min^{-1}	MS/MS-APCI Positive ion mode w/SRM	*Anal. Bioanal. Chem.*, 378, 987, 2004[73]

procedures to utilize a programmable UV/visible detector. The method quantitated vitamins D_2 and D_3 and the previtamin D forms, β-carotene, all-*trans*-retinol, and all-*rac*-α-tocopherol. Detection wavelengths were 265 nm for vitamin D_2 and D_3, 295 nm for the previtamin D forms, 450 nm for β-carotene, 325 nm for retinol, and 295 nm for α-tocopherol. Vitamin K_1 was extracted after lipase hydrolysis and quantitated at 270 nm.

Other studies on margarines and edible oils include work by Seppanen et al.[16] on resolution of β-carotene, tocopherols, and tocotrienols from olive and soybean oils; Psomiadou and Tsimidou[18] on resolution of carotenoids and tocopherols from olive oil; Ye et al.[22] on resolution of tocopherols and β-carotene from reduced-fat mayonnaise, and Puspitasari-Nienaber et al.[29] on the simultaneous assay of carotenoids and tocopherols in vegetable oils; and Stöggl et al.[31] on the simultaneous determination of carotenoids, tocopherols, and γ-oryzanol in crude rice bran oil. The work of Puspitasari-Nienaber et al.[29] combined reversed-phase chromatography on C_{30} and coulometric electrochemical array detection with microsampling procedure developed by Ferruzzi et al.[34] The method used direct injection of 20–30 ng of oil and eliminated saponification and reduced sample preparation time, elimination potential, carotenoid isomerization, and degradation. Detection limits for carotenoids and tocopherols were 1 fmol and 0.15 pmol, respectively, representing a 1000- and 25-fold increased sensitivity over UV/visible detection. The procedure demonstrated the resolution power of the C_{30} support and usefulness for multianalyte fat-soluble vitamin studies. Development of the C_{30} support was discussed in Chapter 1.

Stöggl et al.[31] compared C_{18} and C_{30} supports for their ability to resolve carotenoids, tocopherols, and γ-oryzanol in crude rice bran oil. Higher resolution was found for all analytes with the C_{30} support. Further, the study applied atmospheric pressure chemical ionization mass spectrometry (MS-APCI) detection to characterize the resolved γ-oryzanol components. Chromatographic resolution of β-carotene, the tocopherols, and γ-oryzanol is shown in Figure 14.1.

14.1.2 *Milk and infant formula*

Fluid milk is fortified with vitamin A and vitamin D. Long-standing recognition of analytical problems associated with the analysis of these vitamins, particularly vitamin D, and the need for rapid, precise methods led to much method development research for their analysis from milk and infant formula. The low concentration of vitamin D, even in fortified products, continues to pose analytical problems. The low concentration and poor detection properties of the vitamin usually require analyte concentration or extensive cleanup procedures that limit application of procedures developed or applicable to other fat-soluble vitamins.

The first simultaneous assay for vitamin A and vitamin D was reported in 1979.[35] The procedure combined aspects of methods developed by Thompson and Maxwell[32] and Henderson and Wickroski.[36] One milliliter of milk was saponified, and the vitamins were extracted with hexane. After evaporation to dryness, the residue was dissolved in methanol and injected onto a Vydac C_{18} column and eluted with a mobile phase of acetonitrile:methanol (90:10). Retinol and vitamin D were assayed at 325 and 265 nm, respectively, by detectors in series. The Association of Official Analytical Chemists (AOAC) International does not provide an official method for the simultaneous analysis of vitamin A and vitamin D in milk or infant formula. Few other methods have been published that deal with the specific analysis of vitamin A and vitamin D from milk or infant formula. Methods that assay addition vitamins with vitamin A and vitamin D are discussed later in the chapter.

Analysis of vitamin A activity together with vitamin E activity in milk and infant formula poses fewer problems compared to inclusion of vitamin D in the analysis. Landen[4] adapted the nondestructive fat-soluble vitamin isolation technique by gel permeation chromatography[2] to the simultaneous analysis of retinyl palmitate and all-*rac*-α-tocopheryl

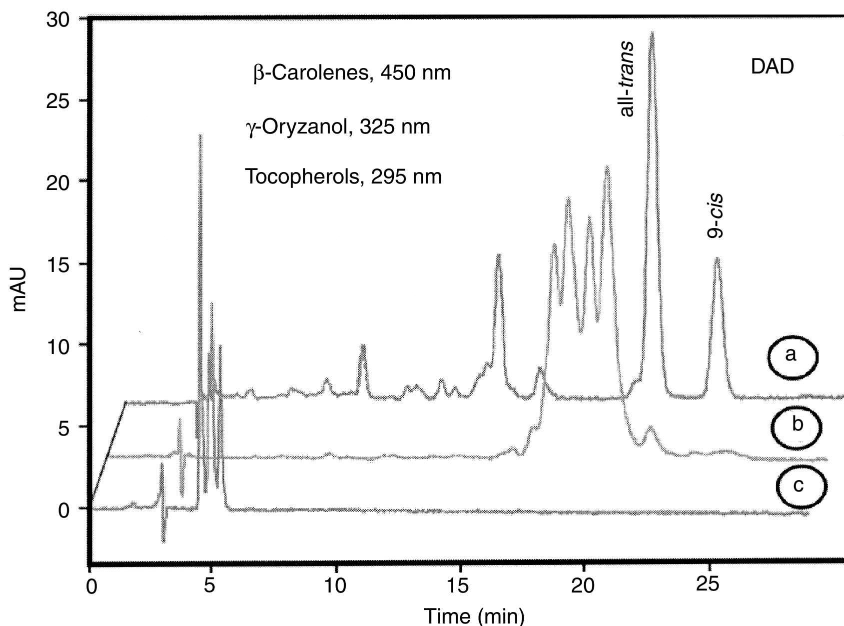

Figure 14.1 Simultaneous determination of (a) β-carotene, (b) γ-oryzanol, and (c) α-, β-, γ-, δ-tocopherol. Chromatographic conditions: stationary phase, Bischoff Prontosil 200-3-C30 (3 μm, 200 Å, 250 × 2 mm id); mobile phase, methanol:*tert*-butyl methyl ether (75:25 v/v); flow rate, 1 mL min^{-1}; detection, UV, (1) 450 nm, (2) 325 nm, (3) 295 nm; temperature, 25°C; injection volume, 5 μL. (Reproduced from Stöggl, W., Huck, C., Wongyal, S., Scherz, H., and Bonn, G., *J. Sep. Sci.*, 28, 1712, 2005. With permission.)

acetate in infant formula. Application of the technique originally developed for margarine analysis resulted in the development of a lipid extraction procedure utilizing a mixture of isopropanol and methylene chloride. After initial extraction steps, $MgSO_4$ was added to remove water. This extraction procedure is applicable to other matrices in addition to fluid milk and infant formulas. Landen's modified procedure[4] added a third μStyragel to the HP-GPC cleanup system. The third gel permeation column provided essentially complete lipid removal from the fat-soluble vitamin fraction, eliminating rechromatography of the isolated vitamin fraction. Methanol (2 mL) was added per liter to the mobile phase of methylene chloride:acetonitrile (30:70). Addition of methanol to the solvent system for C_{18} chromatography improves peak resolution and eliminates peak tailing caused by exposed SiOH groups on the bonded stationary phase.[37] After reversed-phase chromatography, retinyl palmitate, and all-*rac*-α-tocopheryl acetate were simultaneously detected using two detectors in series set at 313 nm for retinyl palmitate and 280 nm for all-*rac*-α-tocopheryl acetate. Overall, the nondestructive approach developed by Landen and his colleagues presented an integrated system that was used for several years at the Atlanta Center for Nutrient Analysis (ACNA), Southeast Regional Laboratory, Food and Drug Administration. The approach was useful for assay of foods fortified with all-*trans*-retinol esters, vitamins D_2 and D_3, all-*rac*-α-tocopheryl acetate, β-carotene, and vitamin K_1; in addition, natural tocopherols, α-carotene, and β-apo-8′-carotenal could be quantified. Primary applications include analysis of retinyl palmitate and β-carotene in margarine,[2] retinyl palmitate and all-*rac*-α-tocopheryl acetate in infant formula[4,5] and breakfast cereals,[3] and vitamin D and vitamin K_1 in infant formula and fortified milk.[6] The analysis system was used to assay fortified foods, medical foods, and pharmaceuticals.

The approach is centered on gel permeation chromatography (HP-GPC) for lipid removal and isolation of the fat-soluble vitamin fraction. The combination of HP-GPC and nonaqueous reversed-phase (NARP) chromatography has the following advantages, which were incorporated into future method development studies by various investigators:

1. The nondestructive extraction used small volumes of solvent and required no heat or saponification. Potential for vitamin degradation is decreased and production of previtamin D forms is minimized.
2. The fat-soluble vitamins were completely resolved from the lipid fraction by HP-GPC.
3. Vitamin A levels in the sample could be estimated before the quantitative step by monitoring absorbance of HP-GPC eluates at 313–325 nm.
4. β-carotene, vitamin A esters, and vitamin E esters were assayed simultaneously following HP-GPC cleanup with NARP chromatography.
5. Modification of the NARP mobile phase allowed resolution of α-, γ-, and δ-tocopherols.

Quantitation of vitamin D and vitamin K required an additional cleanup step on μBondapak-NH_2 to remove UV absorbing interferences and plant sterols in infant formula and cholesterol in milk from the fat-soluble vitamin fraction isolated by HP-GPC. The integrated system shown in Figure 14.2 is an efficient analytical approach to the analysis of

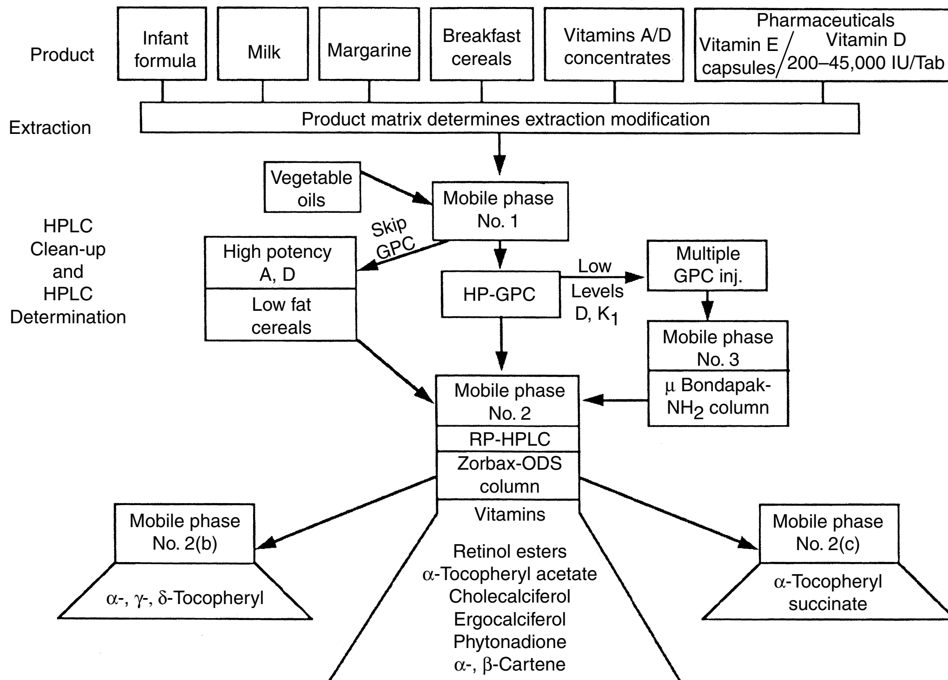

Figure 14.2 Integrated system for quantification of fat-soluble vitamins. Mobile phase compositions: No. 1—methylene chloride, No. 2 (a)—acetylnitrile:methylene, chloride:methanol (700:300:2), No. 2 (b)—acetylnitrile:methylene chloride:methanol (700:300:50), No. 2 (c)—acetylnitrile:methylene chloride (700:300) containing 2% v/v acetic acid, No. 3—methylene chloride:isooctane:isopropanol (600:400:1).

products fortified at varying levels. Most advantageous to regulatory analysis, the nondestructive extraction quantitates the specific forms of the vitamins added in the formulations. Problematic to regulatory activities, the procedure was not collaborated through AOAC International.

Several useful simultaneous methods have been developed for milk and infant formula assay that have the capability to assay more than two fat-soluble vitamins. A nonaqueous reversed-phase chromatography system with two Zorbax ODS columns in series was utilized by Barnett et al.[1] to resolve all-*trans*-retinol, retinyl palmitate, vitamin D_2 or vitamin D_3, *RRR*-α-tocopherol, all-*rac*-α-tocopheryl acetate, vitamin K_1, and cholesterol phenyl acetate (IS). Gradient elution with methanol:ethyl acetate (86:14) against acetonitrile achieved baseline resolution of the analytes. A microprocessor-controlled variable wavelength UV detector monitored the elution. Retinol was monitored at 325 nm and the other analytes were monitored at 365 nm. The extraction procedure was based upon lipid removal by hydrolysis with lipase from *Candida cyclindraccae*. Free fatty acids were removed from the hydrolysis mixture by alkali precipitation. Vitamins were extracted with *n*-pentane following fatty acid removal. The elimination of saponification avoids isomerization of vitamins D_2 and D_3 and destruction of vitamin K_1. Although the chromatography run time was in excess of 50 min, the relative standard deviations (RSDs) for all analytes were less than 5%. The Barnett et al.[1] method provided another approach to nondestructive extraction, which allows the analysis of vitamin K_1 together with other fat-soluble vitamins. Previous methods[2,33] and the lipase hydrolysis technique are not only useful for inclusion of vitamin K_1 and D_2 and D_3 in multianalyte methodology but also provide for assay of specific esters of retinol and α-tocopherol from fortified foods.

Zamarreño et al.[26–28] used electrochemical detection for the simultaneous analysis of fat-soluble vitamins in milk and milk powder. Amperometric detection with a glassy carbon electrode at +1050 mV gave low ng detection limits. The earlier method[26] assayed vitamin D_3 in unenriched milk without preconcentration. The 1995 method[27] assayed vitamin K_1, all-*trans*-retinol, and *RRR*-α-tocopherol in milk after extraction by lipase hydrolysis. Hexane extraction after saponification was used in the procedure published in 1992,[26] which destroyed vitamin K_1. The overall method was adapted to an online system for the simultaneous analysis of all-*trans*-retinol, α-tocopherol, and vitamin D_3 from milk and powdered milk.[28] The online system linked saponification, preconcentration, and cleanup on C_{18} Sep Pak and injection onto a Brownlee OD-224 RP18 column.

Recent method development studies for analysis of fat-soluble vitamins in infant formulas have advantageously used narrow-bore columns[25] and MS detection.[30] Chávez-Seruin et al.[25] assayed retinyl acetate, retinyl palmitate, α-tocopheryl acetate, and α-, γ-, and δ-tocopherol in milk-based infant formulas with normal-phase LC on a Pinnacle II silica short narrow-bore column (5 cm × 2.1 mm) with diode array detection. Extraction of the analytes was by the addition of ethanol to reconstituted infant formula, followed by hexane partitioning. The hexane phase could be directly injected. Precision, recovery, and sensitivity parameters showed the assay to be highly reliable and reproducible.

Heudi et al.[30] developed a normal-phase LC method with positive atmospheric pressure chemical ionization mass spectrometry (LC-MS-APCI) for assay of all-*trans*-retinol, vitamin D_3, and α-tocopherol in fortified infant formula. Internal standards for the procedure were vitamin D_2 and 5,7-dimethyl tocol. A saponification procedure used in official European Committee for Standardization (CEN) methods as described in Chapters 1 (vitamin A), 2 (vitamin D_3), and 3 (vitamin E) was used to hydrolyze ester forms of the vitamins. The analytes were isolated from the saponification digest by solid-phase extraction (SPE) using a Chromabond XTR, 70 mL cartridge. Application of the LC-MS-APCI system gave results comparable to those obtained by LC-UV and LC-fluorescence methods. Within-day and between-day measures of precision were excellent (%CV of 2.3–5.9 and 2.4–6.9, respectively).

The limits of detection were below 1.4 ng. The authors concluded that "the use of LC coupled to a single quadruple MS instrument shows considerable potential for routine analysis of fat-soluble vitamins in infant formula."

14.1.3 Pharmaceuticals

Multianalyte methods for pharmaceutical matrices are summarized in Table 14.2.[38–47] Scalia et al.[38] successfully used supercritical fluid extraction (SFE) to isolate retinyl palmitate and all-*rac*-α-tocopheryl acetate from cream and lotions. SFE conditions were 30 min with supercritical carbon dioxide at 40°C and 250 atm. Recoveries greater than 91.6% were achieved. The SFE minimized sample handling and the use of harmful solvents. The extracted vitamins were collected at the restrictor into 4 mL of THF:methanol (4:1) in a glass vial held at 0°C. The contents of the vial were diluted to 5 mL and directly injected into the LC. Chromatography was completed on a μBondapak C_{18} column and eluted with methanol: acetonitrile (75:25). A programmable UV/visible detector monitored retinyl palmitate at 325 nm and all-*rac*-α-tocopheryl acetate at 280 nm.

Blanco et al.[48] applied narrow-bore (2.1 mm i.d.), small particle (3 μm) C_{18} columns to the simultaneous analysis of all-*trans*-retinol, vitamin D_2, all-*rac*-α-tocopheryl acetate, and vitamin K_1 in pediatric parenteral solutions. The narrow-bore columns gave detection limits of less than 1 ng on-column for retinol, vitamin D_2, and vitamin K_1, which are lower than most obtained with conventional columns. For all-*rac*-α-tocopheryl acetate, the detection limit was 4.85 ng, which is excellent for UV detection. A multichannel UV/visible detector was used with wavelengths set at 325 nm (retinol), 265 nm (vitamin D_2), 284 nm (α-tocopheryl acetate), and 250 nm (vitamin K_1). Mobile phase was 100% methanol pumped at 0.2 mL min^{-1}. Resolution was obtained within 13 min. The narrow-bore columns resulted in significant savings in mobile phase and, thus, cost savings.

Li and Chen[43] presented a method that shows the versatility of diode array detectors for the assay of high concentration multivitamins. Their procedure simultaneously assayed 12 analytes including 9 water-soluble vitamins, retinyl acetate, vitamin D_3, and α-tocopheryl acetate. Tablets were extracted with ethanol for the fat-soluble vitamins and the residue was then extracted with phosphate buffer to obtain the water-soluble vitamins. The two extracts were combined and diluted with mobile phase (ethanol:water, 50:50). Resolution of the 12 analytes used gradient elution from a μBondapak C_{18} column. This was the first report of methodology capable of simultaneous assay of fat- and water-soluble vitamins from multivitamin preparations.

Applications of LC-MS and LC-MS/MS to fat-soluble vitamin assays in botanicals and multivitamin supplements were recently published.[46,47] Hao et al.[46] quantified α-tocopherol, β-carotene, β-crytoxanthin, lutein, and zeaxanthin in various botanical matrices (oils, dry powders, juices, hard tissues) by normal-phase chromatography and MS/MS-APCI. The procedure was rapid, and complete resolution of the analytes provided high precision and accuracy. Identification of α-tocopherol and the four carotenoids was accomplished by comparing the LC retention time and selected reactant monitoring (SRM) analysis of the sample peaks with certified standard peaks. Quantification was by total ion counts of responding external standards. LC-MS-APCI chromatograms are shown in Figure 14.3.

Similar to studies on other matrices, use of the C_{30} support can add increased resolution and speed to the analysis. Breithaupt and Kraut[47] assayed retinol, retinyl acetate, retinyl palmitate, α-tocopherol, α-tocopheryl, and coenzyme Q_{10} using reversed-phase chromatography on C_{30} with diode array detection. The run time for the six analytes was 28 min with gradient elution. Peak identification was with MS-APCI in the positive ion mode. Limit of quantitation values were ≤2.0 μg mL^{-1} except for α-tocopherol (10 μg mL^{-1}). As discussed in Chapter 3, vitamin E ionizes poorly in the positive ion mode compared to detection with

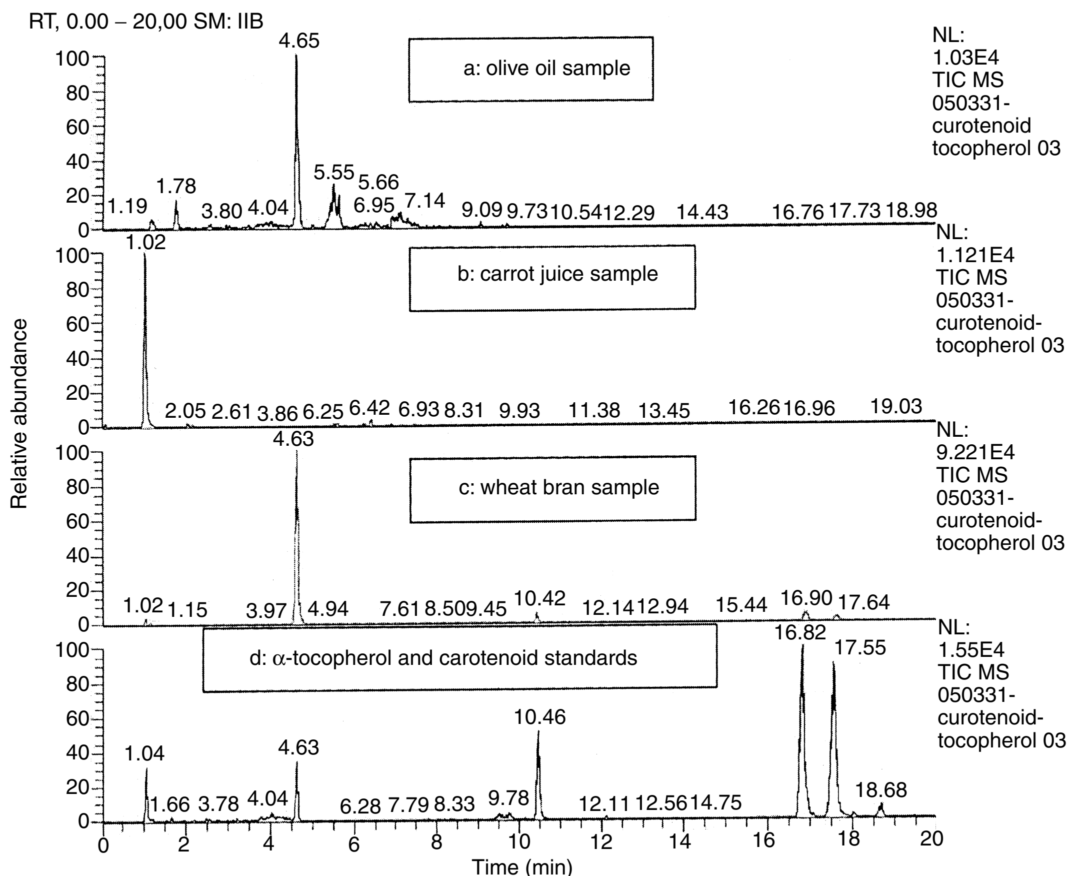

Figure 14.3 LC-APCI-MS chromatograms of (a) olive oil sample (peak at 1.19 min is lycopene), (b) carrot juice sample, (c) wheat bran sample and α-tocopherol, and (d) carotenoid standards. (1) β-Carotene (RT = 1.04 min), (2) α-tocopherol (RT = 4.61 min), (3) β-cryptoxanthin (RT = 10.46 min), (4) lutein (RT = 16.81 min), and (5) zeaxanthin (RT = 17.57 min). (Reproduced from Hao, Z., Parker, B., Knapp, M., and Yu, L., *J. Chromatogr. A*, 1094, 83, 2005. With permission.)

electrospray ionization in the negative ion mode. The authors stated that the method provides unequivocal identification of the vitamins and that the work represented the first application of LC-PDA and LC-MS-APCI to simultaneous analysis of fat-soluble vitamins on the C_{30} support.

14.1.4 Serum and tissues

Methodology for simultaneous analysis of fat-soluble vitamins in clinical samples continues to advance with improvements in detector sensitivity, availability of narrow-bore columns, small particles supports, and better computerization with versatile data management systems. Availability of electrochemical detectors provided increased sensitivity to methods previously based on UV/visible detection. Clinical chemists efficiently used multichannel UV/visible detectors and highly sensitive PDA detectors to further advance simultaneous assay of multiple analytes in serum and plasma. The availability of LC-MS instrumentation, presently, represents another advancement in analytical capability for multianalyte methods for fat-soluble vitamin analysis of serum and other biological materials.

Methodology for simultaneous assay of fat-soluble vitamins in serum or plasma is summarized in Table 14.3.[49–73]

Simultaneous methods for all-*trans*-α-retinol and *RRR*-α-tocopherol in plasma and serum were developed in 1979 by Bieri et al.[49] and De Leenheer et al.[74] Both procedures used C_{18} reversed-phase chromatography with methanol or methanol:water (95:5) mobile phases with UV detection. Internal standards were retinyl acetate,[49] all-*rac*-α-tocopheryl acetate,[49] and tocol.[33] The Bieri et al.[49] method quantitated *RRR*-α-tocopherol, α-tocopheryl quinone, and β-+γ-tocopherol. Ethanol was used to denature blood proteins and the vitamins were extracted with hexane. The detection limits were <100 μg L^{-1} for retinol and <0.8 mg L^{-1} for *RRR*-α-tocopherol. Various studies have modified the original procedures since their introduction, but improvements have been inconsequential to their overall usefulness. Details of the Bieri et al. procedure is provided in Table 14.3.

Fluorescence detection provides increased sensitivity for quantitation of retinol and vitamin E homologs. Rhys Williams[75] incorporated a programmable fluorescence detector to serum analysis for all-*trans*-retinol and *RRR*-α-tocopherol. The sensitivity was such that 25–50 μL of serum could be assayed compared with 200 μL samples normally used for procedures based upon UV detection. With the programmable detector, the α-tocopherol, which eluted at 2.0 min from the normal-phase silica column, was monitored at Ex $\lambda = 295$, Em $\lambda = 390$. After 2.5 min, the wavelengths were changed to Ex $\lambda = 325$, Em $\lambda = 480$ to monitor retinol.

Representative of these methods, Sowell et al.[57] used multiwavelength detection to assay all-*trans*-retiniol, four retinyl esters, *RRR*-α- and *RRR*-γ-tocopherol, all-*trans*-β-carotene, 13-*cis*-β-carotene, α-carotene, lycopene, lutein, β-cryptoxanthin, and zeaxanthin in serum. The method was based on the use of nonapreno-β-carotene and retinyl butyrate as internal standards. The procedure clearly shows the power of an LC system based upon current detector capability and an advanced data handling system (Maxima Software, Waters, Inc.) to simultaneously assay several fat-soluble vitamins and related analytes. Karpińska et al.,[62] likewise, used multiwavelength detection to assay all-*trans*-retinol (324 nm), α-tocopherol (292 nm), and coenzyme Q_{10} (276 nm) in plasma after resolution on LiChrospher 100 RP-18 with an isocratic mobile phase of methanol:hexane (72:28).

A PDA detector can be aligned in series with a fluorescence detector to complete simultaneous assays using UV and fluorescence properties of the analytes. Casal et al.[64] applied normal-phase LC on LiChrosorb Si60 with gradient elution (hexane–isopropanol) to resolve retinol, β-carotene, and α-tocopherol in adipose tissue. A PDA in series with a fluorescent detector was used to quantify the analytes (β-carotene, 452 nm; retinol, 327 nm; α-tocopherol, fluorescence with Ex $\lambda = 295$, Em $\lambda = 330$). The method was rapid, sensitive, and used a low-volume extraction employing ethanol with ascorbic acid (10 mg mL^{-1}) and hexane containing 2% isopropanol and 0.01% *tert*-butyl hydroquinone (TBHQ). The extraction procedure yielded higher concentrations of analytes compared to methods incorporating saponification as the initial treatment. Use of ascorbic acid and TBHQ as antioxidants proved necessary to eliminate oxidative degradation of the analytes.

In early work, Khachik et al.[76] showed the versatility of PDA detection in a study of plasma carotenoids and carotenoid oxidation products. Eighteen carotenoids were resolved and identified using reversed-phase chromatography on C_{18} and chromatography on silica-based nitrile columns. Carotenoids were identified by mass spectroscopy with an LC-MS system interconnected by a particle beam interface. The study represents one of the most intense identification and quantitative studies completed on carotenoids in plasma. Further, it was one of the first applications of mass spectroscopy linked to LC to establish identity of the fat-soluble analytes in plasma. In addition, the research quantified retinol and α- and γ-tocopherol. Carotenoids identified in the plasma by Khachik et al.[76] are given in Table 14.4.

Table 14.4 Carotenoids Identified in Human Serum and Their Absorption Maxima

Plasma carotenoids[a,b]	Absorption maxima (nm)[c]
Eluent B[d]	
ε,ε-Carotene-3,3′-dione	420, 442, 472
3′-Hydroxy-ε,ε-carotene-3-one	(422–424), 442, 472
(all-*E*)-5,6-Dihydroxy-5,6-dihydro-ψ,ψ-carotene (5,6-dihydroxy-5,6-dihydrolycopene)	436, 460, 492
(*Z*)-5,6-Dihydroxy-5,6-dihydro-ψ,ψ-carotene	434, 458, 490
3-hydroxy-β,ε-Carotene-3′-one	(422–424), 448, 476
(*Z*)-3-hydroxy-β,ε-Carotene-3-one	(418–420), 442, 470
(all-*E*,3*R*,3′*R*)-β,ε-Carotene-3,3′ diol ((all-*E*)-lutein)	(422–424), 448, 476
(all-*E*,3*R*,3′*R*)-β,β-Carotene-3,3′ diol ((all-*E*)-3′-zeaxanthin)	(428), 454, 482
(all-*E*,3*R*,3′S,6′*R*)-β,ε-Carotene-3,3′ diol ((all-*E*)-3′-epilutein)	(422–424), 448, 476
(9*Z*)-Lutein	334, 420, 442, 470
(9′*Z*)-Lutein	332, (420), 444, 472
(all-*E*,3*R*)-8′-apo-β-carotene-3,8′ diol (internal standard)	(408), 427, 454
(*Z*,3*R*)-8′-Apo-β-carotene-3,8′ diol	(405), 424, 450
(13*Z*)-Lutein + (13′*Z*)-lutein	334, (418), 440, 468
(9*Z*)-Zeaxanthin	340, (424), 450, 474
(13*Z*)-Zeaxanthin	338, (419), 446, 470
(15*Z*)-Zeaxanthin	338, (426), 450, 478
Eluent A[e]	
(all-*E*)-3-Hydroxy-2′,3′-didehydro-β,ε-carotene ((all-*E*)-2′,3′-anhydrolutein)	(424), 446, 474
(*Z*)-2′,3′-Anhydrolutein	332, (420), 440, 468
Ethyl β-apo-8′-carotenoate (IS)	444, (468)
β,ε-Caroten-3-ol (α-cryptoxanthin)	(424), 446, 476
3-Hydroxy-β-carotene (β-cryptoxanthin)	(428–430), 454, 480
(all-*E*)-ψ,ψ-Carotene ((all-*E*)-lycopene)	446, 472–474, 502
(*Z*)-ψ,ψ-Carotene ((*Z*)-lycopene)	346, 360, 442, 468, 498
7,8-Dihydro-ψ,ψ-carotene (neurosporene)	(420–422), 440, 468
β,ψ-Carotene (γ-carotene)	(440), 462, 492
7,8,7′,8′-Tetrahydro-ψ,ψ-carotene (ζ-carotene)	378, 400–402, 426
β,ε-Carotene (α-carotene)	(428), 446–448, 474
(all-*E*)-β,β-Carotene	(430), 454, 478
(*Z*)-β,β-Carotene	334, (422), 446, 474
(all-*E*)- or (*Z*)-7,8,11,12,7′,8′-Bexahydro-ψ,ψ-carotene ((all-*E*)- or (*Z*)-phytofluene)	332–334, 350, 368
7,8,11,12,7′,8′,11′,12′-Octahydro-ψ,ψ-carotene (phytoene)	(276), 286, (295)

[a] (*Z*)-Carotenoids have been designated the same number as their all-*E*-isomers, but distinguished from their all-*E*-compounds by prime symbols.
[b] Common names for certain carotenoids are shown in parentheses.
[c] Values in parentheses represent points of inflection.
[d] Isocratic—Hex:CH_2Cl_2:MeOH:*N*,*N*-diisopropylethylamine (74.65:25:0.25:0.1).
[e] MeCN (45%):CH_2Cl_2 (22.5%):Hex (22.5%):MeOH (10%).

Source: Khachik, F., Beecher, G. R., and Goli, M. B., Separation and identification of carotenoids and their oxidation products in the extracts of human plasma, *Anal. Chem.*, 64, 2111, 1992.

The advantages of increased sensitivity and ability to unequivocably identify and characterize fat-soluble analytes by LC-MS add a new dimension to clinical chemistry. Kurilich et al.[72] determined bioavailability of lutein, β-carotene, retinol, and phylloquinone from kale intrinsically labeled with carbon-13. The kale tissue was uniformly labeled with ^{13}C by growing plants continuously in an atmosphere containing $^{13}CO_2$. Application of

LC-MS-APCI quantified lutein, β-carotene, retinol, and phylloquinone in the serum of a subject fed a single serving of the intrinsically labeled ^{13}C-kale. Clearly defined kinetic curves depicting concentrations of ^{13}C-lutein, ^{13}C-β-carotene, ^{13}C-retinol, and ^{13}C-phylloquinone in the serum were obtained. The LC-MS-APCI analysis showed the subject ingested 33 μmol of labeled lutein, 34 μmol of labeled β-carotene, and 156 mmol of labeled phylloquinone. Peak plasma concentrations represented 3% (lutein), 1% (β-carotene), and 7% (phylloquinone) of the dose. The LC-MS-APCI analysis allowed simultaneous and selective detection of the ^{13}C-labeled and unlabeled analytes.

Andreoli et al.[73] developed an LC-MS/MS-APCI method for assay of retinol, β-carotene, and α-tocopherol in serum. Detection limits were 10–20× better for the analytes compared to other studies using MS. Positive ion MS/MS-APCI product-ion mass spectra of retinol, β-carotene, and α-tocopherol are shown in Figure 14.4. The authors concluded

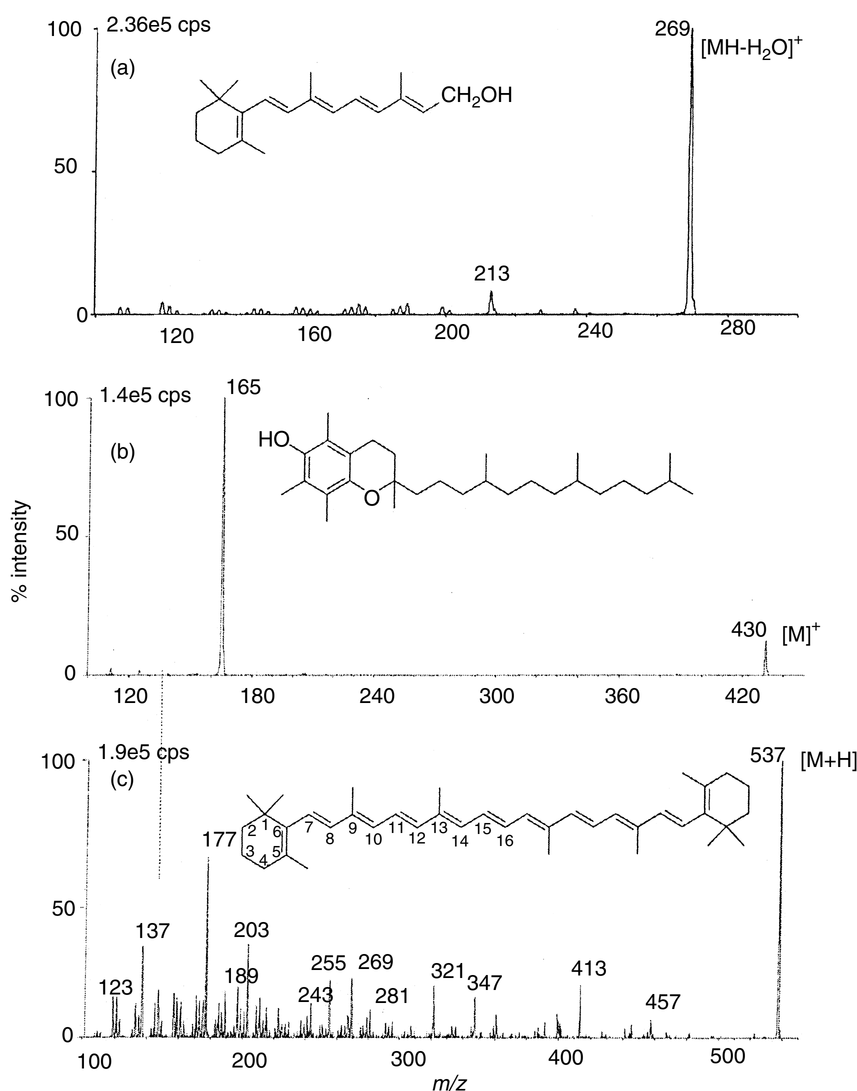

Figure 14.4 Flow-injection PI-APCI-MS/MS product-ion mass spectra of (a) retinol, precursor ion $[MH\text{-}H_2O]^+$, *m/z* 269, (b) α-tocopherol, precursor ion $[M]^+$, *m/z* 430, and (c) β-carotene, precursor ion $[M+H]^+$, *m/z* 537. (Reproduced from Andreoli, R., Manini, P., Poli, D., Bergamaschi, E., Nutti, A., and Niessen, W. M., *Anal. Bioanal. Chem.*, 378, 987, 2004. With permission.)

that "the peculiar characteristics of high selectivity, specificity, and sensitivity, specific for MS detection, allowed a significant overall simplification of the extraction procedure of the analytes from the serum matrix." Without a doubt, LC-MS capability adds greatly to all phases of vitamin chemistry, biochemistry, and analysis.

14.2 Water-soluble vitamins

Development of multianalyte methods for analysis of water-soluble vitamins in nonfortified foods and most biologicals must overcome difficulties of low natural levels of the vitamins, presence of multiple metabolic forms, sometimes specific to the matrix, substantial differences in physical properties, and potential for the vitamins to be complexed with macromolecules such as transfer proteins or, less specifically, to complex carbohydrates. The relatively low concentrations of water-soluble vitamins in natural products, therefore, requires magnitudes of greater sensitivity of the detection system compared to sensitivity required for higher concentration supplements, premixes, or fortified foods. Likewise, specific extraction methods are required for each water-soluble vitamin to maximize extraction from complex matrices. In general terms, multianalyte methods are quite easily developed for pharmaceuticals, supplements, and vitamin premixes, and much harder to perfect for biologicals including serum, tissues, and foods. Owing to analytical problems associated with the aforementioned demands placed on water-soluble vitamin assays, few multianalyte methods exist for biologicals and nonfortified foods compared to those available for fortified foods, vitamin premixes, and supplements. Similar to the situation noted for multianalyte assay of the fat-soluble vitamins, LC-MS instrumentation is a new tool that can significantly improve detection sensitivity and selectivity. LC-MS will undoubtedly impact availability of multianalyte methods for low concentration natural products.

Tables 14.5 and 14.6 present selected multianalyte methods available in the literature for food, serum, and tissue,[77–100] and multivitamins and vitamin premixes.[101–114]

14.2.1 Extraction procedures for multianalyte methods

Extraction procedures developed over several decades continue to be refined for use with determinative methods based on LC resolution. Most extractions used today for the water-soluble vitamins can be traced back to early chemical and/or microbiological assays. Because of variable chemical properties and stability problems, few extractions can be applied to large groups of the various water-soluble vitamins. Some extractant requirements for specific vitamins include the following:

1. For ascorbic acid, the extraction medium must be acidic to maximize stability. *Meta*-phosphoric acid is the most commonly used stabilizer. *Meta*-phosphoric acid is not compatible with many mobile phase systems.
2. Thiamin is stable in acidic media and is the least stable of the water-soluble vitamins as the extractant approaches neutrality. It is unstable in alkaline environments. Extraction methods are designed to quantitate total thiamin or thiamin and the thiamin phosphate esters.
3. Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are hydrolyzed to free riboflavin in acid environments. Therefore, acid hydrolysis must be avoided if quantitation of the coenzymes is required.
4. Niacin is stable in both acid and alkali environments. Measurement of total niacin in cereals requires alkaline hydrolysis.
5. Preservation of phosphorylated forms of vitamin B_6 requires mild extraction conditions. Likewise, acid extractions hydrolyze the glucoside forms of vitamin B_6, which overestimates available vitamin B_6.

Table 14.5 Selected LC and LC-MS Methods for Multi-Analyte Assay of Water-Soluble Vitamins in Food and Other Biological Samples

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	References
Vitamins B_1 and B_2 only				
Potatoes/ vitamins B_1, B_2	Heat and agitate 20 g sample w/65 mL 0.1 M HCl, 30 min. Cool, digest sample solution w/Takadiastase, 45–50°C, 2 h. Dilute to 100 mL w/water. Filter. Derivatize the filtrate to thiochrome w/potassium ferricyanide and NaOH	Waters μBondapak C_{18}, 25 cm × 4.6 mm Mobile phase—isocratic Water:MeOH (70:30) Flow rate—2.0 mL min^{-1}	Fluorescence Ex λ = 365 Em λ = 435 for B_1 Ex λ = 450 Em λ = 510 for B_2	*Food Chem.*, 15, 37, 1984[77]
Dietetic foods/ vitamins B_1, B_2	Heat (water bath) 5 g finely ground sample w/65 mL 0.1 M HCl, 100°C, 30 min. Cool, adjust pH to 4.5 and digest sample w/β-amylase and Takadiastase, 37°C, overnight. Dilute to 125 mL w/water. Filter. Derivatize the filtrate w/potassium ferricyanide and NaOH Clean-up: Sep Pak C_{18}	Waters μBondapak C_{18} Mobile phase—isocratic MeOH:0.05 M NaOAC, pH 4.5 (60:40) Flow rate—1.0 mL min^{-1}	Fluorescence Ex λ = 366 Em λ = 435 for B_1 Ex λ = 422 Em λ = 522 for B_2	*J. Micronutr. Anal.*, 5, 269, 1989[78]
Cereal products/vitamins B_1, B_2	Mix 5 g homogenized sample w/30 mL 0.1 N HCl, autoclave at 125°C, 15 min. Adjust pH to 4–4.5 and add Claradiastase, incubate at 50°C, 3 h. Add 1 mL 50% TCA, incubate further at 90°C, 15 min. Adjust pH to 3.5, dilute to 50 mL, filter. React 10 mL filtrate w/1% potassium ferrocyanide in 15% NaOH Clean-up: Sep Pak C_{18}	μBondapak C_{18}, 10 cm × 8 mm Mobile phase—isocratic MeOH : 5 mM phosphate buffer, pH 7 (35:65) Flow rate—0.8 mL min^{-1}	Fluorescence Ex λ = 360 Em λ = 425 for B_1 Ex λ = 440 Em λ = 520 for B_2	*J. Food Compos. Anal.*, 6, 299, 1993[79]
Selected foods/ vitamins B_1, B_2	Mix sample w/0.1 N HCl, autoclave at 121°C, 30 min. Cool, adjust pH to 4.5. Filter. React filtrate w/1% potassium ferricyanide in 15% NaOH, vortex. Let it stand for 1 min. Add 3.75 N HCl Clean-up: Sep Pak C_{18}	μBondapak C_{18}, 30 cm × 3.9 mm Mobile phase—isocratic MeOH:5 mM NH_4OAC, pH 5 (28:72) Flow rate—1.5 mL min^{-1}	Fluorescence Ex λ = 370 Em λ = 435 for B_1 Ex λ = 370 Em λ = 520 for B_2	*J. AOAC Int.*, 76, 1156, 1993[80]
Meat, liver/ vitamins B_1, B_2	Add 35 mL 0.01 M HCl to 5 g homogenized sample, autoclave at 121°C, 30 min. Add Takadiastase suspension, Claradiastase suspension and papain suspension to meat sample, for liver, add Claradiastase suspension. Adjust pH 4.5, incubate at 37°C, 16–18 h. Filter and adjust pH to 6.5. Clean-up: Nucleosil C_{18}	Nucleosil C_{18}, 3 μm, 15 cm × 4.6 mm Mobile phase—isocratic 0.01 M KH_2PO_4 (pH 3): MeCN containing 5 mM sodium heptanesulphonate (84:16) for meat sample (85:15) for liver	UV 254 nm	*J. Chromatogr. A*, 668, 359, 1994[81]

Foods/vitamins B_1, B_2	Add 65 mL 0.1 N HCl to 5 g ground sample, water-bath, 100°C, 30 min. Adjust pH 4.5. Add β-amylase and Takadiastase, incubate at 37°C, 18 h. Dilute to 125 mL w/water. Filter, inject to LC for B_2. For B_1, react 1 mL filtrate w/3 mL potassium ferrocyanide and clean-up using Sep Pak C_{18}	ODS, 10 μm, 25 cm × 4 mm Mobile phase—isocratic MeOH:0.05 M NaOAC (30:70) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 366 Em λ = 435 for B_1 Ex λ = 422 Em λ = 522 for B_2	*Food Chem.*, 56, 81, 1996[82]
Mushrooms/vitamins B_1, B_2	Add 60 mL 0.1 M HCl to 2 g homogenized sample, heat the mixture in the water-bath at 95–100°C, 30 min. Cool, adjust pH to 4–4.5. Add 5 mL 10% Takadiastase solution, incubate at 45–50°C, 3 h. Add 2 mL 50% TCA, heat mixture at 100°C, 5 min. Dilute to 100 mL with water. Filter. Mix 5 mL filtrate w/300 μL 1% potassium ferrocyanate in 15% NaOH, 10 s. Add 100 μL orthophosphoric acid (17%), filter	Spherisorb ODS-2 C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient MeOH–water Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 360 Em λ = 425 for B_1 Ex λ = 422 Em λ = 515 for B_2	*J. Agric. Food Chem.*, 49, 1450, 2001[83]
Sea urchin, marine seaweeds/vitamins B_1, B_2	Heat 2.5 g sample w/15 mL 0.1 N HCl in a water-bath at 100°C, 30 min. Cool, adjust pH, add 1.25 mL 6% aqueous Claradiastase and incubate at 50°C, 3 h. Cool, dilute w/water to 25 mL, filter. Add 1.25 mL 1% potassium ferricyanide in 15% NaOH to 2.5 mL filtrate, stand for 1 min. Add 0.25 mL 85% H_3PO_4 Clean-up: Sep-Pak C_{18}	Spherisorb C_{18}, 5 μm, 25 cm × 4 mm Mobile phase—isocratic MeOH:5 mM NH_4OAC (pH 5) (28:72) Flow rate—1.35 mL min^{-1}	Fluorescence Ex λ = 370 Em λ = 435 for B_1 Ex λ = 370 Em λ = 520 for B_2	*Int. J. Food Sci. Nutr.*, 55, 259, 2004;[84] *J. Chromatogr. Sci.*, 42, 117, 2004[85]
Meats, livers/vitamins B_1, B_2	Heat sample (5 g) w/60 mL 0.1 N HCl in a water-bath at 100°C, 15 min. Homogenize the mixture, 1 min, continue to heat the mixture for another 45 min. Cool, adjust pH to 4.3-4.7, digest the mixture w/50 mg acid phosphatase at 37°C, 18 h. Add 2 mL 50% TCA, heat at boiling water, 10 min. Dilute to 100 mL w/water. Filter. For B_1, add 5 mL water saturated isobutanol, 3 mL 1% potassium ferricyanide solution in 15% NaOH to 5 mL sample solution, mix, 15 s. Inject isobutanol layer	Inertsil 5 ODS-2, 20 cm × 3 mm Mobile phase—isocratic MeOH:water (40:60) Flow rate—0.3 mL min^{-1}	Fluorescence Ex λ = 366 Em λ = 434 for B_1 Ex λ = 450 Em λ = 510 for B_2	*J. Food Compos. Anal.*, 19, 831, 2006[86]

Continued

Table 14.5 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	References
Water soluble vitamins				
Beef, pork, lamb/ vitamins B_1, B_2, niacin	Hydrolyze 5 g ground sample w/60 mL 0.1 N HCl and 2 mL 6 N HCl at 121°C, 30 min. Cool, adjust pH to 4–4.5, incubate sample mixture w/5% Takadiastase and 10% papain, 42–45°C, 3 h. Add 2 mL 50% TCA and heat in 100°C water bath, 10 min. Cool, dilute to 100 mL, filter. Convert B_1 to thiochrome by adding 5 mL 1% potassium ferricyamide in 15% NaOH to 10 mL filtrate. Extract thiochrome w/10 mL isobutyl alcohol	Alltech C_{10}, 10 μm, column dimension not mentioned. Mobile phase—isocratic 0.02 M phosphate buffer:MeOH (pH 7.0, 70:30) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 378 Em λ = 430 for B_1 Ex λ = 464 Em λ = 540 for B_2 UV 254 nm for niacin	*J. Agric. Food Chem.*, 36, 1177, 1988[87]
Medical foods, infant formula/vitamins B_1, B_2, B_6	Mix sample w/water, add 2 mL perchloric acid to 60 mL sample mixture, stir, 1 h. Adjust pH to 3.2 ± 0.4. Dilute the sample to 200 mL w/mobile phase. Refrigerate the solutions overnight. Filter	Nova Pak C_{18}, 30 cm × 3.9 mm Mobile phase—isocratic Hexanesulfonic acid—MeCN (pH 3.60) Flow rate—1 mL min^{-1}	Fluorescence Ex λ = 295 Em λ = 395 for B_6 Ex λ = 440 Em λ = 565 for B_2 Ex λ = 360 Em λ = 435 for B_1	*J. AOAC Int.*, 75, 561, 1992;[88] 76, 1276, 1993[89]
Infant formula/vitamins B_1, B_2, B_6, B_{12}, niacin, folic acid	Liquid infant milk—add 1 g TCA solid to 10.5 g sample, stir, 10 min. Centrifuge, add 3 mL 4% TCA to the solid residue obtained, mix. Dilute combined two acid extracts to 10 mL w/4% TCA. Filter Powder infant milk—add 10 mL water to 8 g sample, mix, then prepare the sample as the above	Tracer Spherisorb ODS 2, C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic Water:MeOH (85:15) containing 0.5% TEA, 2.4% glacial HAC, 5 mM octanesulfonic acid (pH 3.6) Flow rate—1 mL min^{-1}	Multi-wavelength	*J. Chromatogr. A*, 778, 247, 1997[90]
Rumen liquor/ vitamins B_1, B_2, B_6, niacin	Hydrolyze 2 mL sample w/5 mL 0.2 N H_2SO_4 on steam-bath, 100°C, 10 min. Add 0.4 mL 5 N NaOAC, digest the solution w/0.02 g diastase, water-bath, 45°C, 20 min. Add 0.4 mL 5 N NaOAC. Centrifuge, filter	Lichropher RP-18, column dimension not mentioned. Mobile phase—isocratic MeOH:water (15:85) containing 5 mM hexane sulphonic acid sodium, 0.5% TEA, 2.4% glacial HAC Flow rate—2 mL min^{-1}	UV 280 nm	*Indian J. Animal Sci.*, 69, 567, 1999[91]

Infant formulas/vitamins B_1, B_2, B_6, niacin	For ready-to-feed formulas use 30 mL. For milk powder, add 30 mL warm water (<40°C) to 6 g sample. Add 30 mL 0.6 M TCA to the solutions, shake, 15 min. Filter	Luna Prodigy ODS 3, 5 µm, 15 cm × 4.6 mm, 15.5% carbon content Mobile phase—isocratic MeOH:water:formic acid (25:74:1) containing 0.1% sodium dioctylsulfosuccinate, pH 2.8 Flow rate—2 mL min^{-1}	Fluorescence Ex λ = 290 Em λ = 390 for B_6 Ex λ = 450 Em λ = 510 for B_2 UV 258 nm for niacin	*J. AOAC Int.*, 85, 945, 2002[92]
Fortified foods/pantothenic acid, folic acid	Extract well ground sample w/extraction buffer (50 mM HEPES and 50 mM CHES containing 2% sodium ascorbate, 20 mM 2-MCE, pH 7.85), stir at 20°C, 1 h. Filter	Aqua C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—gradient 0.1% formic acid—MeCN Flow rate—0.8 mL min^{-1}	MS/MS-ESI in positive ion mode	*Anal. Chim. Acta*, 495, 133, 2003[93]
Baby foods/vitamins B_1, B_2, B_6, B_{12}, niacin, folic acid	Hydrolyze 10 g sample w/25 mL 0.1 M HCl, 90°C (water-bath), 30 min. Cool, adjust pH to 4. Digest the sample w/0.1 g Takadiastase, stir at 50°C (water-bath), 2 h. Add 1 mL 50% TCA, heat at 90°C (water-bath), 10 min. Cool, adjust pH to 6, dilute to 50 mL w/10 mM KH_2PO_4 (pH 6), centrifuge, filter	Supelco RP-Amide C_{16}, 5 µm Mobile phase—gradient 10 mM KH_2PO_4 (pH 6) - MeCN Flow rate—1 mL min^{-1}	Multiwavelength	*J. Chromatogr. A*, 1007, 77, 2003[94]
Royal jelly/vitamins B_1, B_2, B_6	Add 1 mL 8% TCA to 0.5 g sample, vortex and centrifuge, 5 min. Filter	Vydac® C_{18}, 5 µm, 25 cm × 4.6 mm Mobile phase—isocratic Hexanesulfonic acid: NH_4OH:MeCN:water (0.09:0.05:9.02:90.84) (pH 3.6) Flow rate—1.0 mL min^{-1}	Fluorescence Ex λ = 295 Em λ = 395 for B_6 Ex λ = 450 Em λ = 525 for B_2 UV 254 nm for B_1	*J. Braz. Chem. Soc.*, 15, 136, 2004[95]
Turkish food/vitamins C, B_1, B_2, B_6, niacin, folic acid, panthothenic acid	Homogenize 5 g sample w/20 g water, centrifuge. Use 10 mL supernatant for the analysis Clean-up: Sep-Pak C_{18} (500 mg)	Discovery C_{18}, 5 µm, 15 cm × 4.6 mm Mobile phase—isocratic MeOH:0.1 M KH_2PO_4 (pH 7.0) (10:90) Flow rate—0.7 mL min^{-1}	UV/VIS 290 nm 550 nm	*ACTA Chromatographica*, 15, 289, 2005[96]

Continued

Table 14.5 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	References
Water soluble vitamins (*Continued*)				
Milk/vitamins B_2, B_6	Add 0.5 mL 1 M TCA to 5 mL milk sample, mix and incubate, room temperature, 30 min. Centrifuge, 2x. Dilute combine two middle parts to 5 mL w/water. Filter. Dilute 1 mL filtrate to 5 mL w/mobile phase	Phenomenex Luna C_{18}, 5 µm, 25 cm × 3 mm Mobile phase—isocratic (10 mM sodium pentanesulfonate—1% HAC):(98% MeOH—2% THF) (82:18) Flow rate—0.4 mL min^{-1}	Fluorescence detection w/ variable wavelength	*Anal. Chim. Acta*, 538, 135, 2005[97]
Italian pasta/nine water–soluble vitamins	For B_1, B_2, B_6, nicotinamide, pantothenic acid—Acid hydrolyze the ground sample, centrifuge and filter For folic acid—digest ground sample w/α-amylase, centrifuge and filter	Discovery RP-Amide C_{16}, 5 µm, 15 cm × 4.6 mm Mobile phase—gradient 20 mM ammonium formate (pH 3.75)—MeOH Flow rate—0.75 mL min^{-1} (split ratio 1:50).	MS/MS-ESI in positive ion mode w/SRM	*Anal. Chim. Acta*, 531, 87, 2005[98]
Plasma/vitamins B_2, B_6	Add d_2-PLP, d_3-PL, d_8-riboflavin (IS). Precipitate proteins with TCA. Hold on ice 60 min. Centrifuge	Agilent C_8, 3.5 µm, 15 cm × 4.6 mm Mobile phase—gradient A—650 mol L^{-1} HAC B—100 mmol L^{-1} HFBA C—MeOH:water (90:10) Flow rate—1 mL min^{-1}	MS/MS-MRM positive ion mode	*Clin. Chem.*, 51, 1206, 2005[99]
Supplemented foods/eight water-soluble vitamins	For solid sample—add 4.5 g warm water (40°C) to 0.5 g sample, homogenize the mixture, 1 min, sonicate, 5 min. Stand in dark, 60 min. For liquid sample—weigh 5 g homogenized sample Add 1 g precipitation solution to the sample, mix, stand in dark, 15 min. Centrifuge and filter the supernatant	Spherisorb ODS-2, 3 µm, 25 cm × 4.6 mm Mobile phase—gradient MeOH—phosphate buffer (pH 2.95) Flow rate—1 mL min^{-1}	Fluorescence and UV detection w/variable wavelength	*J. Agric. Food Chem.*, 54, 5431, 2006[100]

Table 14.6 Selected LC and LC-MS Methods for Multi-Analyte Assay of Water-Soluble Vitamins in Multi-Vitamins and Premixes

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	References
Pharmaceutical preparations/vitamins B_1, B_2, B_6, B_{12}, niacin, folic acid	Extract sample using electronically controlled extraction apparatus	Vertex LiChrosorb RP-18, 5 µm, 25 cm × 4 mm Mobile phase—isocratic MeOH:water:85% H_3PO_4 (55:45:1) containing 0.00064% octanesulphonic acid Flow rate—1.5 mL min^{-1}	UV 254 nm	*J. Chromatogr.*, 390, 448, 1987;[117] *Analyst*, 112, 989, 1987[101]
Multivitamin formulations/vitamins B_1, B_2, B_6, niacinamide	Mix 15 mL Tris buffer w/2 mL sample solution, digestion the sample w/alkaline phosphatase at 37°C, 30 min. Add one drop monothioglycerol to the sample after the digestion and dilute to 50 mL w/buffer solution, pH 3.6 and filter	Altex Ultrasphere ODS, 5 µm, 15 cm × 4.6 mm Mobile phase—isocratic MeCN:water (10:90) containing TEA, EDTA, heptane sulphonic acid sodium, pH 3.6 Flow rate—1 mL min^{-1}	280 nm	*J. Pharm. Biomed. Anal.*, 6, 87, 1988[102]
Multivitamin premixes/thiamin, riboflavin, pyridoxine, niacin	Dissolve 1 g composite powder w/25 mL 0.1 N HCl, sonicate until clear. Make appropriate dilutions based on label declarations w/mobile phase, and add internal standard (*m*-hydroxybenzoic acid)	Nova Pak C_{18}, 15 cm × 3.9 mm Mobile phase—isocratic Water containing small amount of MeCN, 1-hexanesulfonic acid, NH_4OH Flow rate—1.0 mL min^{-1}	UV 280 nm 254 nm	*J. Micronutr. Anal.*, 7, 15, 1990[103]
Liquid multivitamin solution/niacin, FMN, pyridoxine, thiamine, folic acid, riboflavin	Dilute the sample and filter	Spherisorb ODS-2, 3 µm, 10 cm × 2.1 mm Mobile phase—isocratic 5 mM HSA—02% MeOH—0.1% TEA—0.01 M KH_2PO_4/H_3PO_4, pH 2.8 Flow rate—0.2 and 0.3 mL min^{-1}	UV 254 nm 280 nm	*J. Liq. Chromatogr.*, 17, 1525, 1994[104]
Pharmaceutical preparations/vitamins C, B_2, B_{12}, niacin, folic acid	Mix finely powdered tablet w/water, filter	Lichrosorb RP-18, 5 µm, 25 cm × 4 mm Mobile phase—gradient MeOH—0.05 M NH_4OAC Flow rate—1 mL min^{-1}	UV 270 nm	*J. Liq. Chrom. Rel. Technol.*, 20, 3203, 1997[105]
Pharmaceutical formulations/vitamins B_1, B_2, B_6, B_{12}, niacin	Samples contain water- and fat-soluble vitamins. Use SPE (C_{18}, AR) to separate these two group before analysis	Novapak C_{18}, 4 µm, 15 cm × 3.9 mm Mobile phase—gradient MeOH—0.05 M NH_4OAC Flow rate—1.0 mL min^{-1}	UV 270 nm 362 nm	*J. Chromatogr. A*, 870, 207, 2000[106]
Pharmaceutical preparations/nine water-soluble vitamins	Dissolve tablet in 50 mL 0.1 M phosphate buffer, pH 7. Dilute to appropriate concentration w/the buffer and filter	µBondapak C_{18}, 10 µm, 30 cm × 3.9 mm Mobile phase—gradient MeOH—0.1 M KH_2PO_4, pH 7 Flow rate—1.5 mL min^{-1}	PDA	*J. Sep. Sci.*, 24, 271, 2001[107]

Continued

Table 14.6 (Continued)

Sample matrix/ analyte	Extraction/clean up	Column/mobile phase	Detection	References
Multivitamin tablets/vitamins B_1, B_2, B_6, niacin	Shake sample (fine powder) w/sodium thiosulphate solution and water, 65°C, 15 min. Cool, dilute to appropriate concentration w/water. Filter	Hypersil C_{18}, 5 μm, 15 cm × 4.6 mm Mobile phase—isocratic MeOH:0.5% HAC containing 2.5 mM HSA (18:82) Flow rate—1.2 mL min^{-1}	UV 280 nm	*Biomed. Chromatogr.*, 16, 504, 2002[108]
Multivitamin tablets/vitamins B_1, B_6, B_{12}	Shake finely powdered tablet w/water, 40 min and sonicate, 3 min. Dilute to appropriate concentration w/water. Filter	Hypersil-BDS C_{18}, 3 μm, 10 cm × 4.6 mm Mobile phase—gradient Aqueous 0.015% TEA, pH 2.7—MeCN Flow rate—1.5 mL min^{-1}	UV 280 nm 350 nm	*J. Pharm. Biomed. Anal.*, 30, 1403, 2002[109]
Multivitamin tablets/vitamins B_1, B_6, B_{12}, niacin, folic acid	Mix finely powdered tablet w/15% MeOH in water, sonicate, 15 min. Dilute to appropriate concentration w/same solvent. Filter	Supelcosil ABZ^+, 5μm, 15 cm × 4.6 mm Mobile phase—isocratic MeOH:0.1% HSA, pH 2.8 Flow rate—1.0 mL min^{-1}	UV/VIS 290 nm 550 nm	*J. Serb. Chem. Soc.*, 70, 1229, 2005[110]
Pharmaceuticals/ vitamins B_2, B_6	Capsules—mix sample w/1% HAC. Tablets—dissolve sample w/water, sonicate, 3 min Syrup—dissolve sample w/water. Dilute above solution w/mobile phase for LC analysis	Phenomenex Luna C_{18}, 5 μm, 25 cm × 3 mm Mobile phase—gradient 10 mM sodium pentanesulfonate—HAC—MeOH—THF Flow rate—0.4 mL min^{-1}	Fluorescence detection w/variable wavelength	*Anal. Chim. Acta*, 538, 135, 2005[111]
Pharmaceutical formulations/vitamins B_1, B_6, B_{12}	Mix finely powdered tablet w/water, stand for 40 min. Filter	Supelco C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—isocratic 0.05 M phosphate buffer—10% MeOH—0.018 M TEA, pH 3.55 Flow rate—1 mL min^{-1}	UV EC	*J. Chromatogr. A*, 1094, 91, 2005[112]
Polyvitaminated premixes/vitamins B_1, B_2, B_6, B_{12}, C, niacin, folic acid	Shake well homogenized sample (2 g) w/40 mL water and 4 mL 2 M NaOH. Add 50 mL phosphate buffer 1 M, pH 5.5, dilute to 100 mL w/water. Sonicate, 10 min. Filter	YMC-Pack Pro C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient 0.025% TFA (pH 2.6)—MeCN Flow rate—0.8 mL min^{-1}	UV 210 nm 275 nm	*J. Chromatogr. A*, 1070, 49, 2005[113]
LC-MS				
Multivitamin tablets/vitamins B_1, B_2, B_6, C, niacin, biotin, folic acid, pantothenic acid	Shake finely powdered tablet w/water containing 0.024% ammonia vigorously, 1 min. Sonicate 30 min, 60°C. Cool, adjust pH to 7 w/formic acid. Filter supernatant	Spherigel C_{18}, 5 μm, 25 cm × 4.6 mm Mobile phase—gradient 5 mM HFBA—MeOH Flow rate—1 mL min^{-1} Flow rate into MS—0.2 mL min^{-1}	MS-ESI in positive and negative ion mode	*Anal. Chim. Acta*, 569, 169, 2006[114]

6. Folate is subject to oxidative degradation. Reducing agents must be included in extractants. Multiple enzyme treatments (α-amylase, protease) are required to free bound folates from some food matrices.
7. Vitamin B_{12} is most stable at pH levels approaching neutrality. Extraction conditions must free vitamin B_{12} from the sample matrix and stabilize labile cobalamins.
8. Biotin is usually liberated from food matrices by acid hydrolysis, which cleaves biocytin. However, biotin is subject to oxidation and hydrolysis conditions must be fully evaluated before use on a specific matrix.
9. Pantothenic acid is most stable from pH 5.0 to 7.0. Enzyme digestions are required to free pantothenic acid from CoA and dephosphorylate pantetheine.

14.2.2 Extractions suitable for the simultaneous analysis of thiamin, riboflavin, and vitamin B_6

Two multilaboratory studies were conducted that gave extraction parameters for the concurrent extraction of thiamin and riboflavin. In a French study conducted by the Commission Generale d'Unification des Methods d'Analysis,[82] a procedure originally reported by Hasselmann et al.[78] was used. Hydrolysis with 0.1 N HCl was followed by enzyme digestion with a combination of β-amylase and Takadiastase. The method gave high recoveries except for chocolate powder, which required an increase in HCl concentration to 0.5 N to obtain reliable results.

The European Union Measurement and Testing Program published details of an optimal extraction procedure developed from earlier studies.[115] Steps in the extraction were the following:

1. Autoclave a 0.2–5 g sample in 0.1 N HCl for 30 min at 121°C. Sample size and volume were chosen by laboratories in the study.
2. Adjust an aliquot to pH 4.0 with 4.0 M sodium acetate buffer (pH 6.1).
3. Add 100 mg Takadiastase per g sample.
4. Incubate at 37°C–45°C for 4 h for thiamin (18 h for riboflavin).
5. Cool, filter, or centrifuge.

At this point, the extract was ready for either microbiological or high performance liquid chromatography (HPLC) analysis. This extraction procedure uses a high ratio of enzyme to sample, which could produce high enzyme blanks. Ndaw et al.[116] evaluated the effects of acid hydrolysis combined with α-amylase, protease (papain), and acid phosphatase on the extraction of thiamin, riboflavin, and vitamin B_6 from nonfortified food representing several matrices and certified reference materials. This study showed that hydrolysis with hydrochloride acid was unnecessary when the enzyme digestions were employed.

14.2.3 Milk and infant formulas

Several procedures are summarized in Table 14.5 for analysis of various water-soluble vitamins in infant formula[88–90,92] and fortified milk.[97] The methods are based on ion-pair chromatography with C_{18} supports. Ion-pair reagents were hexanesulfonic acid,[88,89] octanesulfonic acid,[90] dioctylsulfosuccinate,[92] and sodium pentanesulfonate.[97] Woollard and Indyk[92] provided a method for analysis of thiamin, riboflavin, pyridoxine, and niacinamide in supplemented infant formula. The procedure resolved FMN and pyridoxal from the supplemented forms of riboflavin and pyridoxine. Thiamin was independently quantified using a modified mobile phase. Chromatography for the simultaneous assay of FMN, riboflavin, niacinamide, pyridoxal, and pyridoxine is shown in Figure 14.5. Extraction of the vitamins only entailed dissolution with warm water and protein precipitation with trichloroacetic acid. It, therefore, is suitable for routine usage with high sample throughput.

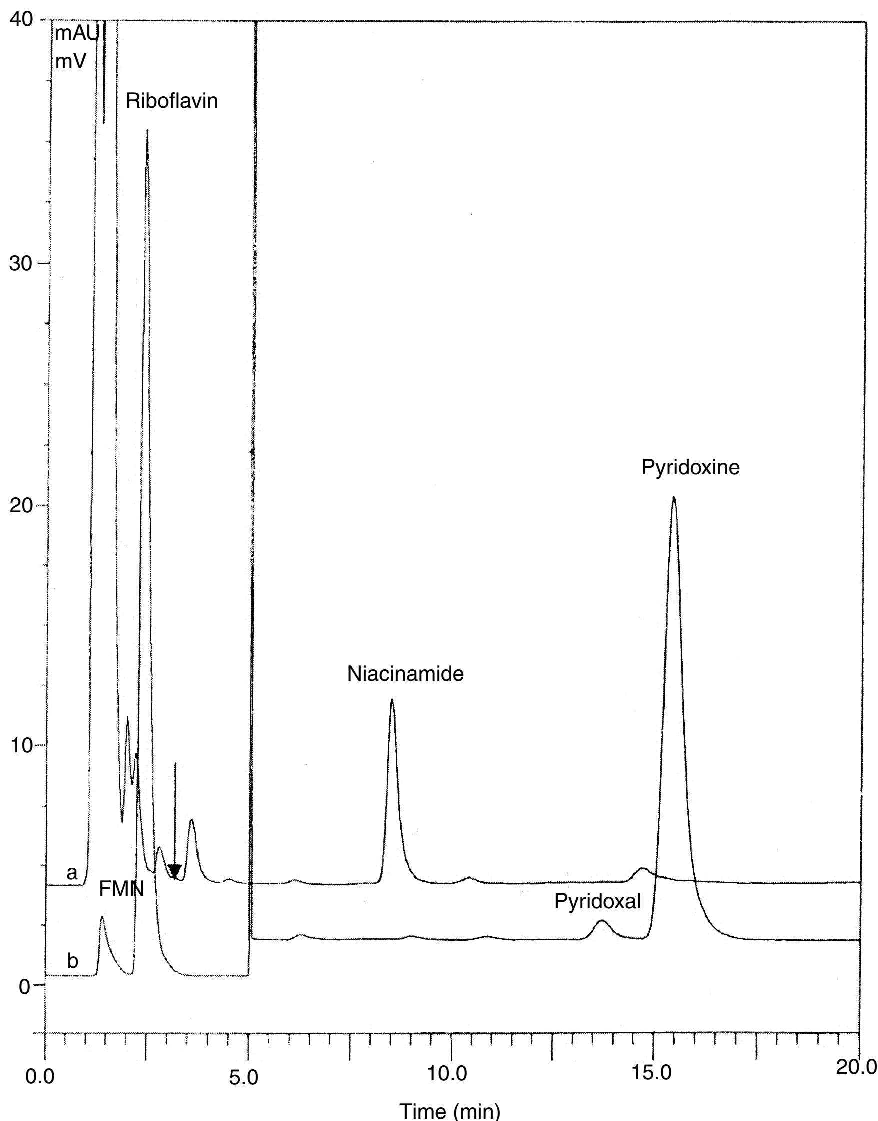

Figure 14.5 Chromatography of milk-based infant formula extract for vitamins B_2 (riboflavin and FMN), B_3 (niacinamide), and B_6 (pyridoxal and pyridoxine). (a) UV at 258 nm; (b) programmed fluorescence (0–5 min, Ex λ = 450, Em λ = 510; 510 nm; 5–20 min, Ex λ = 290, Em λ = 390). Arrow = elution of niacin. (Reproduced from Woollard, D. C. and Indyk, H. E., *J. AOAC Int.*, 85, 945, 2002. With permission.)

Albalá-Hurtado et al.,[90] in earlier work, simultaneously resolved eight water-soluble vitamins from infant formula (nicotinamide, thiamin, riboflavin, pyridoxine, pyridoxal, pyridoxamine, cyanocobalamin, and folic acid). This procedure also incorporated a simple extraction with trichloroacetic acid treatment and centrifugation.

14.2.4 Liquid chromatography-mass spectrometry methods for multi–water-soluble vitamin analysis

Liquid chromatography-mass spectrometry methodology is overcoming many problems associated with determinative steps for quantification of the broad spectrum of fat- and

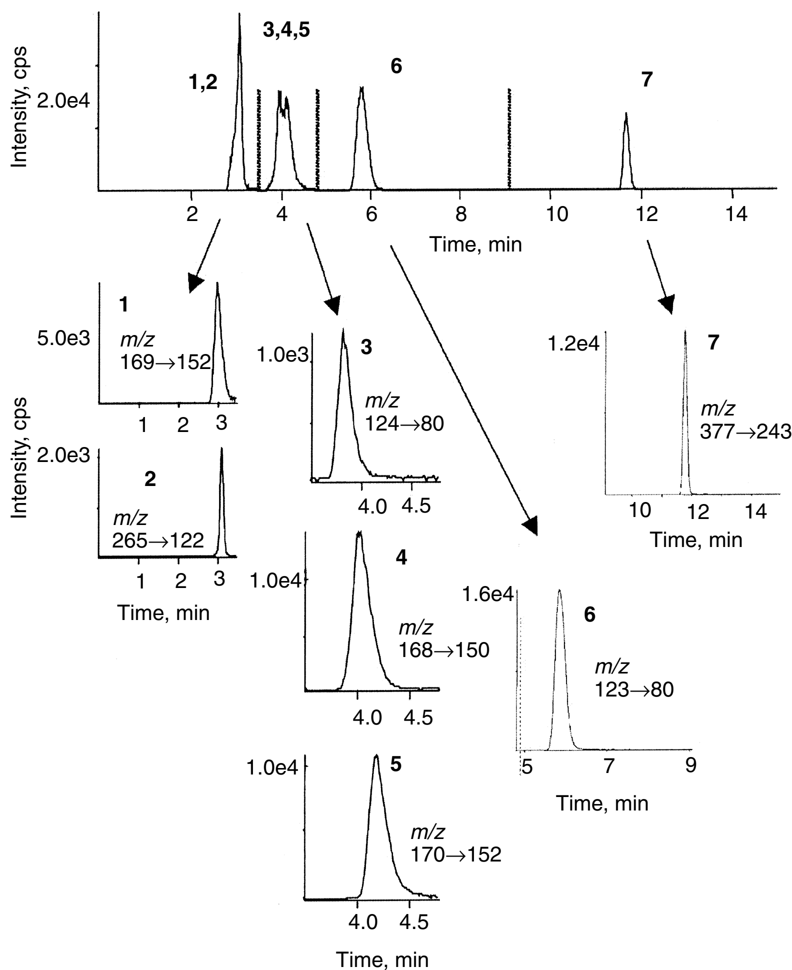

Figure 14.6 LC-ESI-SRM chromatogram of a 1 mg L^{-1} standard mixture containing (1) pyridoxamine (*m/z* 169 → 152), (2) vitamin B_1 (*m/z* 265 → 122), (3) nicotinic acid (*m/z* 124 → 80), (4) pyridoxal (*m/z* 168 → 150), (5) pyridoxine (*m/z* 170 → 152), (6) nicotinamide (*m/z* 123 → 80), (7) vitamin B_2 (*m/z* 377 → 243). (Reproduced from Leporati, A., Catellani, D., Suman, M., Andreoli, R., Manini, P., and Niessen, W. M. A., *Anal. Chim. Acta*, 531, 87, 2005. With permission.)

water-soluble vitamins. Extraction still remains problematic for complex biological matrices. In this regard, working with nonfortified and fortified pasta, Leporata et al.[98] used LC-MS/MS to quantify nine water-soluble vitamins. Thiamin, riboflavin, pyridoxine, pyridoxal, pyridoxamine, nicotinamide, and nicotinic acid were simultaneously assayed. Extraction was by hydrolysis with 0.1 M HCl. The vitamins were characterized by electrospray ionization (ESI) and APCI mass spectra. Positive ion spectra were 100–1000× more intense than negative ion spectra. Limits of detection were in the low µg L^{-1} range. The LC-ESI-SRM chromatogram is shown in Figure 14.6. This overall analysis was sensitive and selective with the capability to be used for assay of water-soluble vitamins in other fortified or nonfortified foods.

The increased sensitivity and selectivity by LC-MS compared to LC procedures with other modes of detection was clearly demonstrated by Midttun et al.[99] A simultaneous assay of all known plasma forms of vitamin B_2 and vitamin B_6 (riboflavin, FMN, FAD, pyridoxal, pyridoxal 5′-phosphate, pyridoxine, pyridoxine 5′-phosphate, pyridoxamine, pyridoxamine 5′-phosphate, and 4-pyridoxic acid) was accomplished by LC-MS/MS in the positive

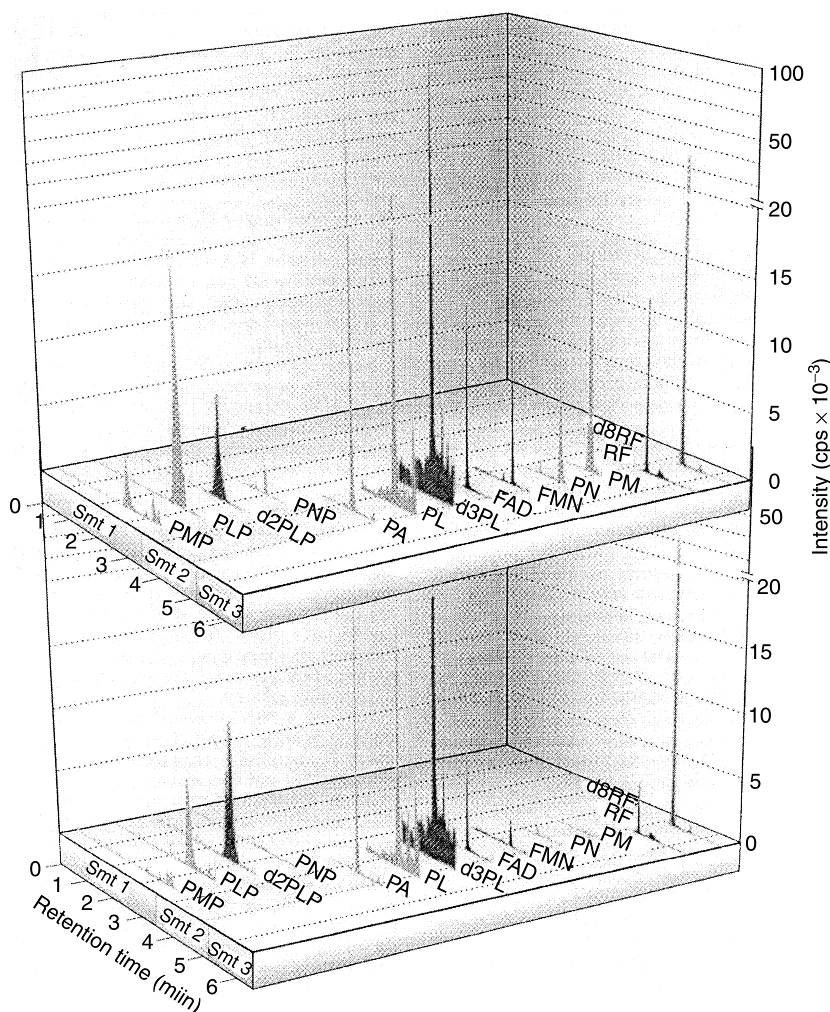

Figure 14.7 LC-MS/MS MRM chromatograms of human plasma containing endogenous vitamin B_6 and B_2 vitamers (top). (Reproduced from Midttun, O., Hustad, S., Solheim, E., Schneede, J., and Ueland, P. M., *Clin. Chem.*, 51, 1206, 2005. With permission.)

ion mode. The analysis procedure used deuterated internal standards and a simple extraction of the plasma with trichloroacetic acid precipitation of the protein and centrifugation. The authors characterized the assay with advantages of simple sample-processing requiring no derivatization and high sample throughput, low sample volume requirements, and short runtimes. Chromatography required 8 min on a C_8 column with gradient elution (Figure 14.7).

14.2.5 Multivitamins and premixes

Method summaries for multianalyte analysis of water-soluble vitamins in multivitamins and premixes are provided in Table 14.6.[101–114,117] Analysis of high concentration products presents few problems compared to analysis of low, natural levels found in most biologicals. Detector sensitivity usually does not pose a problem. The relatively simple matrix decreases chromatographic interferences often seen with biologicals, and the sensitivity of

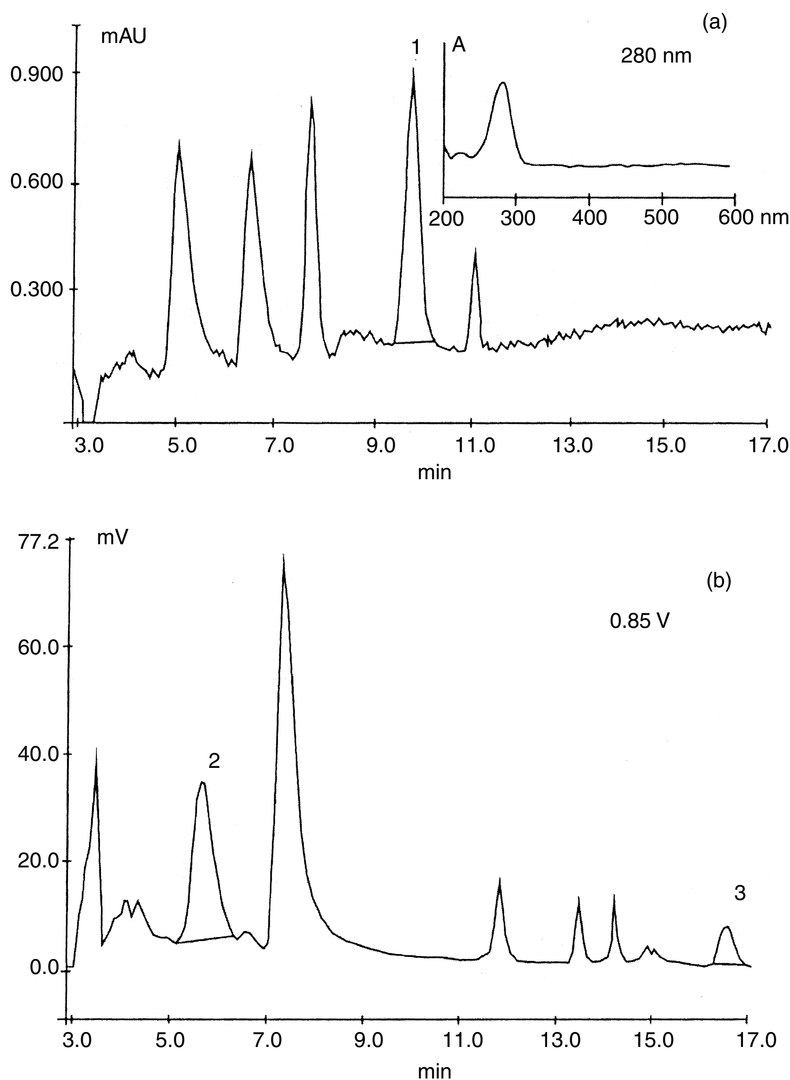

Figure 14.8 Chromatograms of the pharmaceuticals (Visolvit): (a) 1, thiamin (t_R 9.9 min, 1.43 µg mL^{-1}); (b) 2, pyridoxine (t_R 16.2 min, 0.01 µg mL^{-1}). LC 18 column, 5 µm (4.6 mm × 25 cm) Supelco. Mobile phase: 0.05 M phosphate buffer (pH 3.55) with 10% methanol containing 0.018 M trimethylamine, flow rate 1.0 mL min^{-1}. (Reproduced from Marszall, M. L., Lebiedzinska, A., Czarnowski, W., and Szefer, P., *J. Chromatogr. A*, 1094, 91, 2005. With permission.)

UV detection is usually sufficient. Electrochemical detection of multiple vitamins resolved chromatographically also can provide interference-free chromatograms and greater sensitivity compared to UV and fluorescence detection.[112] Figure 14.8 shows the work of Marzall et al.[112] for the simultaneous assay of thiamin (detected by UV at 280 nm) and pyridoxine and cyanocobalamin (detected by coulometric detection). Detectors were in series and reversed-phase chromatography was on a C_{18} support with isocratic elution.

For simple matrix and highly concentrated premixes, UV detection with a photodiode detector can give excellent results. Heudi et al.[113] simultaneously assayed nine water-soluble vitamins (thiamin, vitamin C, nicotinamide, pyridoxine, pantothenic acid, folic acid, cyanocobalamin, riboflavin, and biotin) from premixes used by the infant formula industry.

The vitamins were resolved on YMC-Pack Pro C_{18} column by gradient elution. The mobile phase consisted of 0.025% trifluoroacetic acid, pH 2.6 (solvent A), and acetonitrile (solvent B). The resolution was rapid within 17 min. Thiamin, vitamin C, nicotinamide, pyridoxine, folic acid, and riboflavin were detected at 275 nm, and biotin, pantothenic acid, and cyanocobalamin at 210 nm. Of significance, the mobile phase was chosen for the study because of its compatibility with MS detection. Many mobile phases used in conjunction with multianalyte water-soluble vitamin analyses contain phosphate as a component, making the mobile phase unsuitable for MS detection. Heudi et al.[113] stated that mobile phases containing water and acetonitrile are fully compatible with MS detection and that their current method, with LC-MS, could easily be used to follow formation of vitamin degradation products.

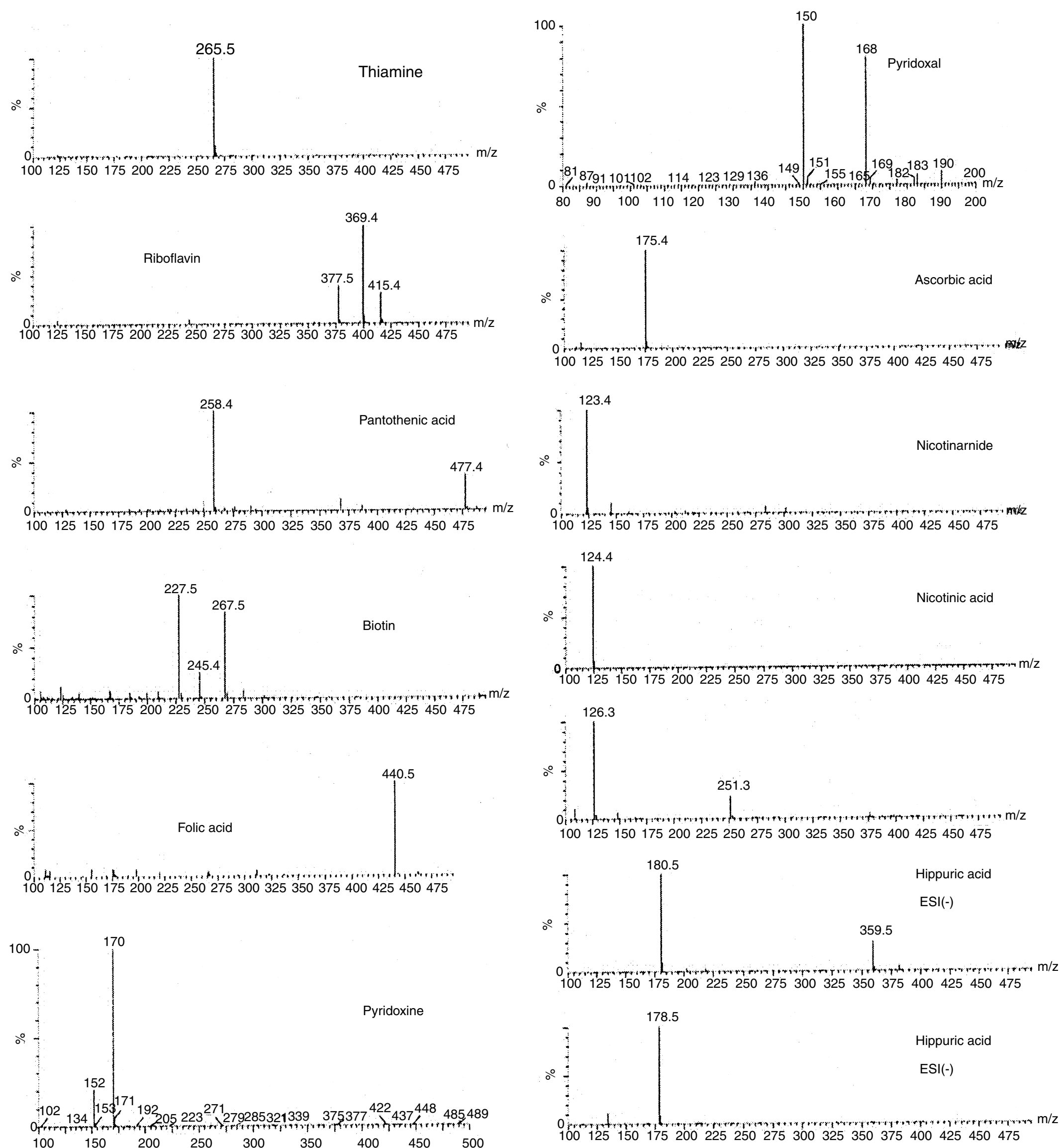

Figure 14.9 MS-ESI spectra of ten water-soluble vitamins, taurine, and hippuric acid (IS) resolved from multivitamins. (Reproduced from Chen, Z., Chen, B., and Yao, S., *J. Agric. Food Chem.*, 46, 5132, 1998. With permission.)

While the sensitivity provided by LC-MS is not necessary for highly concentrated products, it has been adapted for multivitamin assay. Chen et al.[114] resolved ten water-soluble vitamins and taurine from multivitamin tablets on Sperigel C_{18} with detection by MS-ESI with switching continuously from the negative ion mode to the positive ion mode. Hippuric acid was chosen as the internal standard owing to its stable response in both positive and negative ion mode. ESI-MS spectra are given in Figure 14.9.

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Food Science and Technology

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